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Osteopontin contributes to effective neutrophils recruitment, IL-1β production, and apoptosis in *Aspergillus fumigatus* keratitis

Running title: Role of OPN in *A. fumigatus* keratitis

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Abstract

Fungal keratitis is a major cause of corneal ulcers, resulting in significant visual impairment and blindness. A phosphorylated glycoprotein secreted by immunocompetent cells, osteopontin (OPN) mediates cluster formation of the host fungal receptors and enhances the phagocytosis and clearance of pathogenic fungi. However, whether OPN production and function occurs in fungal keratitis is unknown. OPN expression in *Aspergillus fumigatus* keratitis patient corneas was assessed by quantitative polymerase chain reaction (qRT-PCR) and immunofluorescence. Human neutrophils, THP-1 macrophages, and corneal epithelial cells (HCECs) stimulated with *A. fumigatus* were utilized for *in vitro* experiments. Mouse models of *A. fumigatus* keratitis were developed by intra-stromal injection for *in vivo* experiments. Using siRNAs, neutralizing antibodies, recombinant proteins and inhibitors, the production and role of OPN in *A. fumigatus* infection was assessed by clinical evaluation, qRT-PCR, immunofluorescence, western blotting and bioluminescence image acquisition. We observed increased corneal OPN expression in *A. fumigatus* keratitis patients and mouse models compared to controls. OPN production in response to *A. fumigatus* infection was dependent on LOX-1 and Erk1/2. Compared to controls, OPN knockdown impaired pro-inflammatory cytokine IL-1β production, which was dependent upon 4E-BP1. OPN knockdown decreased MPO levels and resulted in decreased neutrophil recruitment, higher

fungal load, and increased apoptosis in mouse *A. fumigatus* keratitis. Our results indicate that OPN is a critical component of the antifungal immune response and is essential for effective neutrophil recruitment, inflammatory cytokine production and apoptosis in *A. fumigatus* keratitis.

INTRODUCTION

The incidence of fungal keratitis is increasing worldwide, and the aetiological agents most commonly responsible are filamentous *Aspergillus* species (*A. fumigatus, A. flavus*) and *Fusarium* (*F. solani, F. oxysporum*) [1]. The impact of fungal keratitis on visual health accounts for up to 60% of corneal ulcers attributable to fungal infection in developing countries, including China, India and Mexico [1-3]. Even in developed countries, such as the United States, fungal keratitis accounts for up to 35% of all corneal ulcers, resulting in severe visual impairment and blindness [1, 4]. Although new therapies are being used in clinical application, fungal keratitis remains a challenge to ophthalmologists because of delayed diagnosis and a lack of effective drugs and treatment methods [5].

Innate immunity is the first line of defence against pathogenic fungi during fungal keratitis. Pattern recognition receptors (PRRs) and C-type lectin receptors (CLRs), for example, dendritic cell-associated c-type lectin-1 (dectin-1) and lectin-type oxidized LDL receptor 1 (LOX-1), play important roles in antifungal immunity [1, 6]. In host immune defence against *Pneumocystis*, cluster formation of antifungal receptors, including dectin-1, Toll-like receptor 2 (TLR2) and mannose receptor (MR), is dependent on osteopontin (OPN), an

adaptor molecule in TLR2 and Dectin-1 signalling pathways mediates ERK activation and cytokine production and enhances the phagocytosis and clearance of pathogenic fungi [7].

OPN, encoded by the secreted phosphoprotein 1 (SPP1) gene, is expressed in a range of immune cells, including macrophages, neutrophils, dendritic cells, and T and B cells, where it acts as an immune modulator in a variety of ways [8-11]. First, OPN has chemotactic properties that promote cell recruitment to inflammatory sites [12, 13]. It also functions as an adhesion protein involved in cell attachment and wound healing [14, 15]. In addition, OPN mediates cell activation and cytokine production, as well as promoting cell survival by regulating apoptosis [16-19]. Furthermore, OPN is involved in granuloma formation and in modulating the severity of *Paracoccidioides brasiliensis* infection [20]. *In vivo*, OPN-deficient mice show increased susceptibility to low load, but not high load, fungal infection [21]. However, the production and role of OPN in fungal keratitis has not been reported.

This study demonstrates that OPN is increased in the cornea of *A. fumigatus* keratitis patients and a variety of additional cell types that are pivotal anti-fungal infection cells found in the cornea, including corneal epithelial cells, macrophages and neutrophils. Upon *A. fumigatus* infection, OPN production occurs in a LOX-1- and Erk1/2-dependent fashion. OPN knockdown impaired pro-inflammatory cytokine IL-1β production induced by *A. fumigatus* infection, which was dependent on the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). A relatively mild inflammatory response and decreased neutrophil recruitment were observed in response to knockdown of OPN, which might be

related to increased apoptosis in *A. fumigatus*-infected corneas. These data suggest that OPN is a critical component of the antifungal immune response in *A. fumigatus* keratitis.

RESULTS

OPN levels are increased in human *A. fumigatus* keratitis and *A. fumigatus*-stimulated neutrophils, THP-1 macrophages and HCECs

OPN mRNA (Figure 1a) and protein levels (Figure 1b) were significantly higher in the corneas of *A. fumigatus* patients compared to healthy controls. OPN mRNA levels in neutrophils (Figure 1c), THP-1 macrophages (Figure 1e) and HCECs (Figure 1g) were higher after stimulation with *A. fumigatus* for 4 and 16 hours compared to controls. In addition, the OPN protein levels measured by western blotting were elevated in stimulated neutrophils (Figure 1d), macrophages (Figure 1f) and HCECs (Figure 1h) at 16-hours post-*A. fumigatus* infection compared to controls.

OPN levels increase in response to *A. fumigatus* keratitis in mice

Figure 2a shows that at 1 day post-infection, C57BL/6 mice developed significant corneal opacity, which persisted up to 14 days post-infection. With increased neovascularization, corneal inflammation gradually improved. Compared with controls, OPN mRNA levels in mouse corneas were significantly higher after 12-hour *A. fumigatus* infection, peaking at 3 days and persisting up to 10 days post-infection (Figure 2b). OPN protein levels were

elevated in infected mouse corneas at 12 hours compared with controls, which persisted up to 7 days post-infection (Figure 2c). Increased OPN was also confirmed by immunostaining. The results showed that OPN protein expression markedly increased in infected mouse corneas 1 day post-infection compared to controls (Figure 2d).

Aspergillus fumigatus-induced OPN production is dependent on LOX-1 and Erk1/2 and independent of Dectin-1 and JNK

Photographs taken by a slit lamp at 1 day post-infection illustrate the disease response in scrambled control versus $siRNA_{LOX-1}$-treated mice (Figure 3a). LOX-1 silencing was assessed by clinical score (Figure 3b), fungal survival (Figure 3c) and protein expression (Figure 3e), which confirmed efficient gene knockdown. OPN mRNA (Figure 3d) and protein (Figure 3e) levels in mouse corneas with $siRNA_{LOX-1}$ treatment were significantly lower after *A. fumigatus* infection. However, no significant differences were observed in corneal OPN expression of mice treated with $siRNA_{Dectin-1}$(Supplementary figure 1a-e).

The role of LOX-1 and Dectin-1 in OPN production upon *A. fumigatus* infection were confirmed using HCEC *in vitro*. After LOX-1 neutralization, expression of IL-1β protein was significantly down-regulated (Figure 3g), confirming its neutralization function. LOX-1 neutralization decreased the mRNA (Figure 3f) and protein (Figure 3g) expression of OPN. However, no significant differences in OPN expression were observed in mice treated with Dectin-1 neutralization (Supplementary figure 1f, g).

Erk1/2 and JNK functions in OPN production in response to *A. fumigatus* infection were confirmed in mouse models and HCEC experiments. The efficacy of the Erk1/2 inhibitor SCH772984 was confirmed by measuringErk1/2 protein phosphorylation levels *in vivo* (Figure 3i) and *in vitro* (Figure 3k). The Erk1/2 inhibitor decreased mRNA and protein expression of OPN *in vivo* (Figure 3h and i) and *in vitro* (Figure 3j and k). However, no significant differences in OPN expression were observed in mice treated with a JNK inhibitor (Supplementary figure 1h-k).

Disease response after siRNA$_{OPN}$ treatment

Photographs taken by slit lamp at 1 day (Figure 4a) and 2 days (Figure 4f) post-infection illustrate the disease response in scrambled control versus siRNA$_{OPN}$-treated mice. The palliative disease response is represented by a clinical score, which was significantly higher at 1 day (Figure 4b) and 2 days (Figure 4g) in scrambled siRNA-treated controls compared to siRNA$_{OPN}$-treated mice. Compared with scrambled siRNA-treated controls, siRNA$_{OPN}$-treated mice exhibited a higher CFU at 1 day (Figure 4c) and 2 days (Figure 4h). OPN silencing was assessed by mRNA and protein detection at 1 day (Figure 4d, e) and 2 days (Figure 4i, j), which confirmed efficient gene knockdown.

siRNA$_{OPN}$ treatment reduced neutrophil infiltration and MPO activity in *A. fumigatus* keratitis mice

NIMP-R14 was stained in the corneas of siRNA$_{OPN}$ and scrambled siRNA-treated mice at 1 day post-infection (Figure 5a). Positive staining (green) in the corneas of siRNA$_{OPN}$ mice indicated the decreased presence of neutrophils compared to scrambled siRNA-treated control mice. Average bioluminescence, measured by an inflammation probe dropped into the surface of mouse cornea, revealed that corneas of siRNA$_{OPN}$-treated mice exhibited a significant decrease in MPO levels at 1 day post-infection compared to scrambled controls (Figure 5b, c). Traditional MPO detection methods using a colorimetric activity assay showed a similar result (Figure 5d).

OPN-mediated IL-1β secretion in response to *A. fumigatus* infection is dependent on 4E-BP1

Compared to controls, IL-1β protein levels in *A. fumigatus*-infected mouse corneas were significantly higher after 12 hours, persisting up to 10 days post-infection (Figure 6a). IL-1β mRNA (Figure 6b) and protein (Figure 6c) levels in mouse corneas exposed to siRNA$_{OPN}$ treatment were significantly lower after *A. fumigatus* infection. Decreased IL-1β mRNA and protein levels were rescued by the addition of rmOPN (Figure 6d and e). The role of OPN in IL-1β production upon *A. fumigatus* infection was also confirmed in HCEC experiments. OPN neutralization decreased the expression of IL-1β mRNA (Figure 6f) and protein (Figure 6g), which was rescued by the addition of rhOPN (Figure 6h, i).

Compared to scrambled siRNA-treated controls, phosphorylation levels of 4E-BP1 in mouse corneas in response to $siRNA_{OPN}$ treatment significantly decreased after *A. fumigatus* infection (Figure 6c). Decreased IL-1β mRNA and protein levels induced by $siRNA_{OPN}$ treatment were rescued by the addition of a 4E-BP1/eIF4E interaction inhibitor, 4E1RCat (Figure 6j and k). *In vitro* experiments showed a similar result (Figure 6l, m).

$siRNA_{OPN}$ treatment increased levels of apoptosis in *A. fumigatus* keratitis mice

Compared to scrambled siRNA-treated controls, Bcl-2 mRNA levels (Figure 7a) in $siRNA_{OPN}$-treated mouse corneas were significantly decreased. BAX (Figure 7b) and caspase-3 (Figure 7c) increased after infection with *A. fumigatus.* The correlative results obtained from western blotting indicated that phosphorylated Akt and Bcl-2 levels significantly decreased, while BAX, cleaved-caspase-3 and cleaved-caspase-9 were significantly increased with $siRNA_{OPN}$ treatment (Figure 7d). However, cleaved-caspase-8 was not affected by $siRNA_{OPN}$ treatment.

DISCUSSION

This study demonstrates that OPN contributes to effective neutrophil recruitment, inflammatory cytokine production and apoptosis in *A. fumigatus* keratitis mice. These findings indicate that OPN may be a critical component of the antifungal immune response in the cornea. Although the role of OPN in mediating fungal keratitis is still not well

understood, several studies indicate its role in antifungal immunity. For example, Inoue *et al.*[19] found that OPN is essential in mediating the cluster formation of fungal receptors that detect *Pneumocystis* and plays a role as an adaptor molecule in the TLR2 and Dectin-1 signalling pathways. Nishikaku *et al.*[20, 22] found that OPN is involved in granuloma formation and in the severity of *Paracoccidioides brasiliensis* infection. Sato I *et al.*[21] found that OPN-deficient mice exhibited increased susceptibility to low fungal load. Taken together, these observations are consistent with our results that OPN plays a critical role in host antifungal immunity.

In addition, we observed increased expression of OPN in human neutrophils, macrophages and corneal epithelial cells in response to *A. fumigatus* infection. Although the cornea is considered an immune privileged region, it has been well established that macrophage cells reside in the corneal stroma and epithelium [23]. Furthermore, neutrophils were shown to be the first cells recruited to the cornea to fight fungal infection [24]. Human corneal epithelial cells are involved in antifungal immunity by way of releasing antimicrobial peptides, inflammatory cytokines and chemokines [25, 26]. The role of OPN in human neutrophils and macrophages in antifungal immunity has been described, but OPN has not previously been examined corneal epithelial cells solely involved in corneal wound healing [27]. Importantly, our study demonstrates that human corneal epithelial cells participate in antifungal immunity by producing OPN.

Production of OPN upon *A. fumigatus* infection is dependent on LOX-1 but not Dectin-1 expression. This observation is consistent with reports showing that OPN expression increased in wild-type mouse models of ischaemia-reperfusion but to a much smaller extent in LOX-1 KO mice [28, 29]. This finding indicates that the LOX-1 membrane receptor is responsible for initiating the expression of intracellular OPN in order to cope with corresponding signals. These findings also indicate that Erk1/2, not JNK, contributes to the amplification of OPN production upon *A. fumigatus* infection.

Furthermore, compared to the normal course of disease in C57BL/6 mice, knockdown of OPN resulted in a reduced clinical score and impaired fungal clearance. Decreased corneal neutrophil recruitment and MPO activity may be responsible for this. As the peroxidase enzyme converts chloride and hydrogen peroxide into hypochlorous acid during the respiratory burst phase, MPO measured by bioluminescence imaging and colorimetric activity assay is used by the neutrophil to kill fungi and other pathogens. Singh R *et al.*[9] found that reduced neutrophil accumulation in the absence of OPN was accompanied by an increase in bacterial load. Barreno *et al.*[30] also found that following O_3exposure, bronchoalveolar lavage fluid neutrophil levels were significantly reduced in OPN-deficient mice compared with wild-type mice. These findings indicate that OPN plays a critical role in the recruitment of neutrophils during the inflammatory response.

Similar to cellular infiltration, OPN knockdown corneas exhibited impaired pro-inflammatory cytokine IL-1β production compared with control corneas. As a critical mediator of the host response to microbial infections, IL-1β is involved in a variety of cellular activities, including

cell proliferation, apoptosis and differentiation. These results are consistent with reports on *Pseudomonas aeruginosa*-induced bacteraemia in which OPN-KO mice exhibited reduced secretion of pro-inflammatory cytokines, such as interferon-γ, IL-1β, IL-12 and tumour necrosis factor-α [31]. OPN knockdown also decreased 4E-BP1 phosphorylation, and IL-1β decreased by OPN knockdown was rescued by treatment with a 4E-BP1/eIF4E interaction inhibitor. This result is consistent with reports on lung tumourigenesis in which triple mutant OPN was observed to decrease NF-κB activity and 4E-BP1 phosphorylation[32]. Previous studies have also shown phosphorylation of mammalian target of rapamycin (mTOR)/4E-BP1 pathways increased IL-1β expression [33, 34]. 4E-BP1 is one member of a large family of translation repressor proteins that directly interact with the eukaryotic translation initiation factor 4E (eIF4E). The interaction of 4E-BP1 with eIF4E inhibits complex assembly and represses translation. 4E-BP1 is phosphorylated in response to various signals, including UV irradiation and insulin signalling, resulting in its dissociation from eIF4E and activation of cap-dependent mRNA translation.

Infection and apoptosis as a combined inflammatory trigger are best suited for clearing infecting pathogens and repairing damage to host tissue during infection [35]. In this study, OPN knockdown increased mitochondrial-dependent apoptosis in mice with *A. fumigatus* keratitis. Inflammatory immune responses induce necrotic cell death of immunocytes and release damage-associated molecular patterns (DAMPs) to amplify the inflammation. However, apoptosis during infection can shape a suppressive, autoreactive, or protective immune response [36]. OPN seems to play an important physiological role in the efficient development of Th1 immune responses and cell survival by inhibiting apoptosis [37-39]. These

findings indicate that OPN knockdown may reduce inflammation by modulating neutrophils recruitment, decreasing pro-inflammatory cytokines and increasing apoptosis.

In conclusion, our results demonstrate the essential pro-inflammatory role of OPN in *A. fumigatus* keratitis. We support these findings using a range of experimental models from patient corneas, *A. fumigatus* keratitis mice models and cell line experiments.

METHODS

Clinical specimens

Six healthy and six *A. fumigatus* keratitis corneas identified by a combination of morphology and fungal culture were used in this study. The ethics committee of the Affiliated Hospital of Qingdao University approved the corneas for use. The use of corneas has been described in previous publications from our laboratory [6]. The research adhered to the tenets of the Declaration of Helsinki. The research aims and methodologies were thoroughly explained to patients, and informed consent was obtained to collect the samples. Patients with acute or chronic systemic illness or with any form of immunosuppression or topical steroid therapy were excluded from this study.

Preparing *Aspergillus fumigatus*

Aspergillus fumigatus strain 3.0772 was purchased from The China General Microbiological Culture Collection Center (Beijing, China). The strain was cultured for 3–4 days on a

Sabouraud agar. Suspensions of fresh conidia scraped from the surface of the medium were quantified with a haemocytometer and adjusted to a final concentration of 5×10^4 conidia μL^{-1} in PBS.

***In vitro* experiments**

Human neutrophil experiments were obtained from the peripheral blood of healthy volunteers and was approved by the ethics committee of the Affiliated Hospital of Qingdao University. Neutrophils were isolated by Ficoll Paque Plus (TBD, Tianjing, China) and suspended in a RPMI-1640 media (HyClone, Logan, UT, USA) at a density of $1\times10^6 mL^{-1}$. Giemsa staining showed >95% neutrophils.

THP-1 macrophages purchased from the China Center for Type Culture Collection (Wuhan, China) were differentiated with 100 nM of Phorbol 12-Myristate 13-Acetate (PMA) (Sigma, St. Louis, MO, USA) for 48hours and allowed to recover for 24 hours prior to infection. Macrophages were cultured in RPMI-1640 medium at a density of 1×10^6 mL^{-1}.

Human corneal epithelial cells (HCECs) were provided by the Ocular Surface Laboratory at the Zhongshan Ophthalmic Center. Cells were cultured to 80% confluence in serum-free DMEM (HyClone, Logan, UT, USA) for 24 hours.

To study OPN expression, human neutrophils, THP-1 macrophages and HCECs were treated with *A. fumigatus* conidia in 12-well and 6-well plates at a multiplicity of infection (MOI) of 1 for 0, 1 and 4hours (qRT-PCR) and 0, 1/4, 1/2, 1, 4 and 16 hours (western blotting).

To study *A. fumigatus*-induced OPN expression and the role of OPN in antifungal immunity, HCECs were treated with neutralizing antibodies or inhibitors 2 hours before treatment with conidia. Neutralizing antibodies included a goat LOX-1 neutralizing antibody (R&D, Minneapolis, MN, USA) at a final concentration of 10 μg mL^{-140}, a goat Dectin-1 neutralizing antibody (R&D) at a final concentration of 30 μg mL^{-1}, a goat OPN neutralizing antibody (R&D) at a final concentration of 10 μg mL^{-1}) [41], and a control goat IgG (R&D). Inhibitors included the JNK inhibitor SP600125 (SelleckChem, Houston, TX, USA) (40 μM) [42], the Erk1/2 inhibitor SCH772984 (SelleckChem) (10 μM) [43] and the 4E-BP1/eIF4E interaction inhibitor 4E1RCat (SelleckChem) (50 μM) [44].

HCECs were treated with human osteopontin recombinant protein (rhOPN; R&D Systems) at a final concentration of 180 ng mL^{-1}) [45] or 4E1RC at 1 hour after treatment with the OPN neutralizing antibody. After pretreatment, HCECs were treated with *A. fumigatus* conidia at an MOI of 1 for 0 and 16 hours in 12-well plates for qRT-PCR and 0 and 16 hours in 6-well plates for western blotting.

***In vivo* experiments**

Eight-week-old C57BL/6 female mice were purchased from the Changzhou Cavens Laboratory (Jiangsu, China). Mice were treated in accordance with the *Statement for the Use of Animals in Ophthalmic and Vision Research* by the Association for Research in Vision and Ophthalmology (ARVO). Mice were anaesthetized with 8% chloral hydrate. *A. fumigatus* conidia ($0.5\times10^5 \mu L^{-1}$) were released into their corneal stroma, and mice were examined daily

by a slit lamp microscope for corneal opacification and ulceration. Ocular disease was graded using clinical scores ranging from 0 to 12 according to the scoring system by Wu *et al.* [46].

To study the expression and role of OPN in *A. fumigatus* keratitis, two methods were used for inhibition: (1) siRNA treatment and (2) pharmacological inhibitors. SiRNA treatment was used for Dectin-1 (Santa Cruz, Santa Cruz, CA, USA), LOX-1 (Santa Cruz) and OPN (Santa Cruz) inhibition. Inhibitor treatments were used for JNK (SP600125; SelleckChem), Erk1/2 (SCH772984; SelleckChem) and 4E-BP1/eIF4E interaction (4E1RCat; SelleckChem) inhibition. For siRNA treatments, the eye randomly selected from mice received a subconjunctival injection (5 µL) containing 8 µM of siRNA or a non-targeting scrambled sequence (negative control) (Santa Cruz) 1 day and 2 hours before *A. fumigatus* infection [47]. For inhibitor treatments, the eye randomly selected from the mice received a subconjunctival injection (5 µL) containing 40 µM of SP600125, 10 µM of SCH772984, or a DMSO control 1 day and 2 hours before infection [48].To assess the function of OPN and 4E-BP1 in IL-1β secretion upon *A. fumigatus* infection, the randomly selected eye from the mouse received a subconjunctival injection (5 µL) containing 8 µM of OPN siRNA 1 day and 2 hours before infection, and a subconjunctival injection (5 µL) containing 100 µg $mL^{-1}$of mice osteopontin recombinant protein (rmOPN; R&D) or 50 µM 4E1RC 1 hour before infection.

Quantification of *Aspergillus* colony forming units (CFUs)

To assess fungal viability, whole eyes were homogenized under sterile conditions in 1 mL of PBS using a Mixer Scientz-48 (Scientz, Ningbo, China) at 33 Hz for 4 minutes. Subsequently,

serial log dilutions were performed and plated onto Sabouraud dextrose agar plates (BDMS, Cockeysville, MD, USA). Following incubation for 24hours at 37°C, the number of CFUs was determined by direct counting.

Myeloperoxidase (MPO) assay

Corneas were cut at one day p.i. and homogenized in 1.0 mL of 50 mM phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethyl-ammonium. Samples were freeze-thawed and centrifuged, and 100 µL of supernatant was added to 2.9 mL phosphate buffer with O-Dianisidine dihydrochloride (0.167 mg mL^{-1}) and 0.0005% hydrogen peroxide. The absorbency (460 nm) was measured at 30-second intervals for 5 minutes. The slope of the line was determined in relation to the assay units of MPO/cornea.

RNA isolation and qRT-PCR

The mRNA levels of OPN and IL-1β in human neutrophils, THP-1macrophages, HCECs and mouse corneas were detected after infection with *A. fumigatus.* The PCR protocol has been described in previous publications [6]. The primer pair sequences are listed in Supplementary table 1.

Immunofluorescence staining

For tissue immunofluorescence, human corneas and mouse eyes (n = 6/group/time) were embedded in an OCT compound (Tissue-Tek; Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. The immunofluorescence protocol has been described in previous publications [6]. Primary antibodies included 10 μg mL^{-1} rabbit anti-human OPN antibody (ProteinTech, Chicago, IL, USA), a rabbit anti-mouse OPN antibody (ProteinTech) and a rat anti-mouse NIMP-R14 antibody (Santa Cruz). FITC-conjugated goat anti-rabbit antibody (1:200; Bioss, Beijing, China), Alexa Fluor 555-conjugated goat anti-rabbit antibody (1:1000; CST, Danvers, MA, USA,) and Alexa Fluor 488-conjugated goat anti-rat antibody (1:1000; CST) were secondary antibodies.

Western blotting

The western blotting protocol for corneas and cells has been described in previous publications [6]. The primary antibodies are listed in Supplementary Table2. Blots were stained using the following HRP-tagged secondary antibodies purchased from CST.

Bioluminescence imaging and image acquisition and analysis

Bioluminescence images were captured and analysed by the *In Vivo* Imaging System (IVIS) spectrum (Perkin Elmer, Santa Clara, CA, USA). Imaging was performed one day after *A. fumigatus* conidia infection. Fifteen minutes before imaging, mice received an

intraperitoneal injection of 8% chloral hydrate. The luminescent reagent (MPO xenoLightrediJect inflammation probe; Perkin Elmer) was dropped onto the surface of the mouse cornea 7 minutes before imaging. Mice were positioned on the IVIS warming stage in the infected eye lateral decubitus position with the ocular surface directly facing the camera sensor. *In vivo* imaging parameters were determined using standard optimization protocols [49, 50]. Mice were imaged using a field of view 'C' with medium binning for 1 minute. Average bioluminescence at baseline and peak inflammation was compared by paired Student's *t*-test using Prism-5 software. Before *t*-test analysis, the normality present in the differences of paired data was tested by Shapiro-Wilk analysis with failure to reject the null hypothesis.

Statistical analysis

An unpaired two-tailed Student's *t*-test was used to determine the statistical significance of the qRT-PCR, western blotting, clinical score and CFU assays. Data were considered significant at $P \leq 0.05$. All experiments were repeated once to ensure reproducibility, and data from a representative experiment are shown as the mean ± SD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure 1. Osteopontin (OPN) is increased in human *A. fumigatus* keratitis and *A. fumigatus*-stimulated neutrophils, THP-1 macrophages, and human corneal epithelial cells (HCECs). Corneas (n = 6) from *A. fumigatus* patients and healthy donors were subjected to quantitative polymerase chain reaction (qRT-PCR) analysis for OPN (**a**). Then, 5-µm paraffin sections were immunostained to visualize OPN (red) protein expression in corneas from *A. fumigatus* patients and healthy donors (**b**). Magnification ×200. Human neutrophils, THP-1 macrophages, and HCECs were treated with *A. fumigatus* conidia at a multiplicity of infection (MOI) of 1 at 0, 1, 4 and 16 hours for RT-PCR (**c**, **e** and **g**) and 0, 1/4, 1/2, 1, 4 and 16 hours for western blotting (**d**, **f**, **h**). The mean values and standard deviations of two independent experiments are shown. ***P*< 0.01, ****P*< 0.001

Figure 2. OPN levels are increased in *A. fumigatus* keratitis mice. Representative corneal photographs of mice *A. fumigatus* keratitis models developed by intra-stromal injection (1×10^5 conidia/cornea) at 0, 1/2, 1, 2, 3, 5, 7, 10 and 14 days post-infection (**a**). Corneas of six mice euthanized at corresponding times were excised and subjected to RT-PCR (**b**) and western blotting (**c**) analysis for OPN. Then, 7-µm frozen sections were immunostained to visualize OPN (red) protein expression in controls and *A. fumigatus*-infected mouse corneas in 1 day post-infection (**d**). Magnification ×400. The mean values and standard deviations of two independent experiments are shown. ***P*< 0.01, ****P*< 0.001

Figure 3. OPN production upon *A. fumigatus* infection is dependent on Lectin-Type Oxidized LDL Receptor 1 (LOX-1) and Erk1/2. Representative corneal photographs of mice *A. fumigatus* keratitis models at 1 day post-infection pretreated with $siRNA_{LOX-1}$ or scrambled siRNA (**a**). LOX-1 knockdown led to lower clinical scores (**b**) and higher fungal load (**c**). The corneas of mice (6/group) euthanized at 1 day post-infection were excised and subjected to RT-PCR (**d**) and western blotting (**e**) analysis for OPN. Effective silencing of LOX-1 was confirmed by measuring protein levels. HCECs were stimulated with *A. fumigatus* conidia (MOI = 1) for 16 hours after a 2-hour LOX-1 neutralization pretreatment. Cells were subjected to RT-PCR (**f**) and western blotting (**g**) analysis for OPN. Effective neutralization of LOX-1 was confirmed by decreased IL-1β protein levels. Erk1/2 inhibitor (SCH772984) was used for pretreatment *in vivo* and *in vitro*. Mouse corneas and HCECs were subjected to RT-PCR (**h** and **j**) and western blotting (**i** and **k**) analysis for OPN after corresponding Erk1/2 inhibitor pretreatment and *A. fumigatus* infection. The efficacy of inhibitor *in vivo* and *in vitro* was confirmed by measuring phosphorylated Erk1/2. The mean values and standard deviations of two independent experiments are shown. $*P < 0.05$, $***P < 0.001$

Figure 4. Disease response after $siRNA_{OPN}$ treatment. Representative corneal photographs of mouse *A. fumigatus* keratitis models at 1 day (**a**) and 2 days (**f**) post-infection pretreated with $siRNA_{OPN}$ or scrambled siRNA. OPN knockdown led to lower clinical scores at 1 day (**b**) and 2 days (**g**) and higher fungal load at 1 day (**c**) and 2 days (**h**) post-infection. Effective silencing of OPN was confirmed by measuring mRNA levels at 1 day (**d**) and 2 days (**i**) and

protein levels at 1 day (**e**) and 2 days (**j**) post-infection. The mean values and standard deviations of two independent experiments are shown. *P< 0.05, **P < 0.01, ***P< 0.001

Figure 5. siRNA$_{OPN}$ treatment reduces neutrophil infiltration and myeloperoxidase (MPO) activity in *A. fumigatus* keratitis mice. Seven μL frozen sections were immunostained to visualize neutrophil infiltration (green) in *A. fumigatus*-infected mouse corneas pretreated with siRNA$_{OPN}$ or scrambled siRNA (**a**). Magnification ×400. Average bioluminescence from inflammation probe dropped into the surface of *A. fumigatus*-infected mouse corneas indicated that corneas of siRNA$_{OPN}$-pretreated mice exhibited a significant decrease in MPO levels at 1 day post-infection compared to scrambled controls by photographs (**b**) and photon detection (**c**). Corneas of mice (6/group) euthanized at 1 day post-infection were excised and subjected to a colorimetric activity assay for MPO (**d**). The mean values and standard deviations of two independent experiments are shown. **P< 0.01, ***P < 0.001.

Figure 6. OPN-mediated IL-1β secretion in response to *A. fumigatus* infection is dependent on Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Corneas of six *A. fumigatus* keratitis mice euthanized at 0, 1/2, 1, 2, 3, 5, 7, 10 and 14 days post-infection were excised and subjected to western blotting analysis for IL-1β (**a**). *A. fumigatus* keratitis mice (6/group) pretreated with siRNA$_{OPN}$ or scrambled siRNA were euthanized at 1 day post-infection, and their corneas were excised and subjected to RT-PCR (**b**) and western blotting (**c**) analysis. siRNA$_{OPN}$-pretreated mice (6/group) were treated with or without rmOPN before *A. fumigatus* infection and euthanized 1-day post-infection. Corneas were excised and subjected to RT-PCR (**d**) and western blotting (**e**) analysis for IL-1β. An OPN neutralization antibody or IgG control was used to pretreat HCECs before *A. fumigatus* infection. HCECs were subjected to RT-PCR (**f**) and western blotting (**g**) analysis for IL-1β.

RmOPN was used for OPN neutralization antibody pretreated HCECs before *A. fumigatus* infection, and cells were subjected to RT-PCR (**h**) and western blotting (**i**) analysis for IL-1β. A 4E-BP1/eIF4E interaction inhibitor (4E1RCat) was used to treat $siRNA_{OPN}$-pretreated mice and HCECs were pretreated with OPN neutralization antibody before *A. fumigatus* infection. Mouse corneas and HCECs were subjected to RT-PCR (**j** and **l**) and western blotting (**k** and **m**) analysis for IL-1β. The mean values and standard deviations of two independent experiments are shown. **P* < 0.05, ***P*< 0.01, ****P*< 0.001

Figure 7. $siRNA_{OPN}$ treatment increases apoptosis in *A. fumigatus* keratitis mice. Corneas of *A. fumigatus* keratitis mice (6/group) pretreated with $siRNA_{OPN}$ or scrambled siRNA euthanized at 1 day post-infection were excised and subjected to RT-PCR analysis for Bcl-2 (**a**), BAX (**b**) and caspase-3 (**c**) and western blotting analysis for phosphorylated Akt, Akt, Bcl-2, BAX, cleaved-caspase-3, cleaved-caspase-9 and cleaved-caspase-8 (**d**). The mean values and standard deviations of two independent experiments are shown. **P*< 0.05, ***P*< 0.01, ****P*< 0.001

Supplementary figure 1. OPN production upon *A. fumigatus* infection was independent of dendritic cell-associated C-type Lectin-1 (Dectin-1) and JNK. Representative corneal photographs of *A. fumigatus* keratitis mouse models at 1 day post-infection pretreated with siRNADectin-1 or scrambled siRNA **(a).** Dectin-1 knockdown led to lower clinical scores **(b)** and higher fungal load **(c).** Corneas of mice (6/group) euthanized at 1 day post-infection

were excised and subjected to RT-PCR (**d)** and western blotting **(e)** analysis for OPN. Effective silencing of Dectin-1 was confirmed at the protein level. HCECs were stimulated with *A. fumigatus* conidia (MOI=1) for 16 hours after a Dectin-1 neutralization pretreatment for 2 hours. Cells were subjected to RT-PCR **(f)** and western blotting **(g)** analysis for OPN. Effective neutralization of Dectin-1 was confirmed by decreased IL-1β protein levels. A JNK inhibitor (SP600125) was used for pretreatment *in vivo* and *in vitro*. Mouse corneas and HCECs were subjected to RT-PCR (**h** and **j**) and western blotting (**i** and **k**) analysis for OPN after corresponding JNK inhibitor pretreatment and *A. fumigatus* infection. Efficacy of inhibitor was confirmed *in vivo* and *in vitro* by measuring phosphorylated JNK. The mean values and standard deviations of two independent experiments are shown. ***P*< 0.01, ****P*< 0.001

Supplementary figure 2. Average bioluminescence from inflammation probe dropped into the surface of mouse cornea increased corneal MPO level after 1 day post-infection of *A. fumigatus* (left) compared to control (right).