ISG15 in Host Defense Against *Candida albicans* Infection in a Mouse Model of Fungal Keratitis

Chen Dong,[1,2] Nan Gao,[1] Bing X. Ross,[1] and Fu-Shin X. Yu[1]

[1]Departments of Ophthalmology and Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan, United States
[2]College of Biological Engineering, Henan University of Technology, Zhengzhou, Henan, China

Correspondence: Fu-Shin X. Yu, Kresge Eye Institute, Wayne State University School of Medicine, 4717 St. Antoine Boulevard, Detroit, MI 48201, USA; av3899@wayne.edu.

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Purpose. ISG15, a di-ubiquitin-like protein, is critical for controlling certain viral and bacterial infections. We sought to determine if ISG15 plays a role in corneal innate immunity against *Candida albicans* (*C. albicans*) using a C57BL/6 (B6) mouse model of human fungal keratitis.

Methods. Scarified corneas of adult B6 mice were pretreated with TLR5 ligand flagellin and then inoculated with *C. albicans*. The expression of ISG15 and other genes involved in ISG15 conjugation (ISGylation) was determined by real-time PCR. ISG15 expression and distribution in infected corneas were assessed by immunohistochemistry. ISGylation was examined by Western blotting. siRNA knockdown and recombinant ISG15 were used to elucidate the effects of ISG15 on controlling fungal keratitis by clinical scoring, fungal number plate counting, ELISA cytokine determination, and polymorphonuclear leukocytes (PMN) infiltration measurement.

Results. Heat-killed *C. albicans* induced expression of ISG15, and hBD2 was markedly enhanced by flagellin-pretreatment in cultured human primary corneal epithelial cells (CECs). In vivo, *C. albicans* infection induced the expression of ISG15, ISGylation-associated genes (UBE1L, UBCH8, and HERC5), and ISGylation in mouse CECs, all of which were enhanced by flagellin-pretreatment. siRNA knockdown of ISG15 increased keratitis severity, dampened flagellin-induced protection, and greatly suppressed the expressions of ISGylation enzymes, IFN-γ, but not CXCL2 in B6 mouse CECs. Recombinant ISG15, on the other hand, enhanced corneal innate immunity against *C. albicans* and suppressed infection-induced IL-1β, but not IL-Ra expression. ISG15 alone induced the expression of IL-1Ra, CXCL10, and CRAMP in mouse CECs. ISG15 was upregulated and secreted in cultured human CECs in response to challenge in a type 1 IFN-dependent manner.

Conclusions. Our data, for the first time, demonstrate that ISG15 acts as an immunomodulator in the cornea and plays a critical role in controlling fungal keratitis.

Keywords: fungal keratitis, ISG15, innate immunity, cornea

The discovery of Toll-like receptors (TLRs) that recognize conserved structures in pathogens has greatly advanced our understanding of innate immunity[1] and has moved the role of the mucosal epithelia from merely acting as a physical barrier to an important participant of host defense in terms of innate immunity.[2,3] Mucosal epithelial cells including that of the cornea express most of known TLRs and respond quickly when exposed to pathogens or ligands by producing proinflammatory cytokines, defense molecules, and cytoprotective factors.[4] We and other authors have shown that stimulation of mucosal epithelia with TLR ligands, particularly TLR5 ligand flagellin, prior to pathogen exposure induces a strong innate defense and promotes robust resistance to microbial infection in the cornea and other tissues.[5–8] To understand the underlying mechanisms for this robust innate immune protection, we recently performed a genome-wide cDNA array and found that flagellin-pretreatment followed by *Pseudomonas aeruginosa* infection resulted in the upregulation of 890 genes and downregulation of 37 genes.[9] Among these genes, a group of interferon (IFN)-induced genes including IRF1, -7, -9, ISG15, IFITM1, -2, IFIT3, -2, IFI27, and CXCL10 were induced in response to infection, and their expression was further augmented by flagellin-pretreatments. While these genes have been shown to play a role in innate antiviral immunity, their involvements in bacterial or fungal infection were less clear.[10,11]

IFN-stimulated gene of 15 kDa (ISG15) is an ubiquitin-like modifier that is highly induced upon viral infection in many cells including innate immune cells and residential epithelial cells.[12,13] ISG15 can be covalently coupled to many host cellular proteins, a process known as ISGylation, often modulating their functions.[14] Analogous to ubiquitin, ISG15 is covalently conjugated to protein targets via the consecutive action of an E1-activating enzyme (Ube1L),[15] an E2-conjugating enzyme (UbcH8),[16] and a few E3 ligases.[17,18] All enzymes involved in the conjugation process are themselves strongly inducible by type 1 IFNs.[18] Removal of ISG15/UCRP is catalyzed by the deconjugating enzyme UBP43/USP18.[19] Viral infections have been shown to greatly induce ISG15 expression.[20–22] ISG15 modification of virus-derived proteins mediates the antiviral activity of the ISGylation system.[14,23] Selective inactivation of USP18 isopeptidase activity in vivo was found to

enhance ISG15 conjugation and viral resistance.[18] In addition to antiviral activity, ISG15 is also known to be induced by bacteria.[24,25] Interestingly, individuals lacking the ubiquitin-like protein ISG15 were previously reported to show enhanced susceptibility to mycobacterial but not viral infection.[17,26,27] To our knowledge, there were no studies of the involvement of ISG15 in fungal infection, as well as in ocular infection and immunity.

In addition to ISGylation, ISG15 has also been found to be secreted from some cell types and to exert immunomodulatory effects, including stimulation of NK cell expansion, activation, and production of IFN-γ.[28,29] Patients with inherited ISG15 deficiency and other inborn errors of IFN-γ immunity share similar susceptibility to mycobacterial infection, suggesting the lack of secreted ISG15 as an underlying cause for the diseases.[17,26,27] More direct evidence for free ISG15 function came from a recent study of Chikungunya virus infection of neonatal mice; mice lacking ISG15, but not UbE1L, were profoundly susceptible to CHIKV infection, suggesting ISGylation is not required. While it has no effect on viral loads, ISG15 deficiency results in a dramatic increase in proinflammatory cytokines and chemokines, suggesting that ISG15 functions as an immunomodulatory molecule.[30] A recent study identified ISG15-deficient patients who do not yet show immunodeficiency but have abnormal calcification in the brain and suffer from seizures.[31] This study reveals that human intracellular ISG15 prevents IFN-α/β overamplification and auto-inflammation in an ISGylation-independent manner.[31,32] Hence, ISG15 has a multifaceted role in host defense against infection and in preventing IFN auto-inflammation and pathology.[17,32,33] Additional studies are needed to clarify the role of this molecule in diverse pathophysiological conditions.

In this study, based on our cDNA array study, we used the mouse experimental *Candida albicans* (*C. albicans*) keratitis model[34,35] and flagellin-induced protection to assess the role of ISG15 in corneal innate immunity against *C. albicans* infection. We showed that ISG15 was mostly expressed in epithelial cells, especially at an early stage of infection. ISG15 siRNA knockdown increased corneal susceptibility to *C. albicans*. Our results suggest that ISG15 functions as an immunomodulator and plays a protective role in mucosal innate immunity against fungal infection in the corneas.

Materials and Methods

Animals, Fungi, and Infection Procedure

Wild type C57BL/6 mice (female, 8 weeks of age; 20 to 24 grams in weight) were used. Animals were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee of Wayne State University approved all animal procedures.

C. albicans strain SC5314, a clinical isolate capable of producing experimental keratomycosis, was cultured on yeast extract peptone dextrose (YPD) agar (Sigma-Aldrich Corp., St. Louis, MO, USA) for 3 days at 25°C. Colonies were harvested after 3 days of inoculation and diluted in sterile phosphate-buffered saline (PBS) to yield 2×10^5 colony-forming units (CFUs)/μL based on the optical density (OD) at 600 nm, using a predetermined OD_{600} conversion factor of 1 OD = 3×10^7 CFU/mL.[28]

For corneal infection, mice were anesthetized, the pretreated corneas were re-scratched 24 hours postflagellin application, and a 5-μL suspension containing 1×10^5 CFU of *C. albicans* strain SC5314 was applied to the surface of the scarified cornea. The anesthetized mice were kept on a fixed position with the instilled solution remaining on the ocular surface until they awakened (usually >20 minutes).

Flagellin Preparation, Pretreatment

Flagellin was prepared from *P. aeruginosa* strain PA01 as described earlier.[36,37] The bioactivity of each preparation was compared with previous samples for their ability to induce tolerance in cultured human CECs.[38] For flagellin pretreatment, mice (n = 5/group/treatment) were anesthetized with ketamine/xylazine and placed beneath a stereoscopic microscope at a magnification of 40×, and the corneas were scratched gently with three 1-mm incisions using a sterile 26-gauge needle. Purified flagellin (500 ng in 5 μL PBS) or PBS for the control was applied to the injured corneas of the same mice (one serves as the control for the other; the data were presented as the number of eyes). After 24 hours, corneas were excised and processed for ISG15 expression.

Human Corneal Epithelial Cell (HCEC) Culture and Treatment

Primary HCECs were isolated from human donor corneas obtained from Midwest Eye-Banks (Ann Arbor, MI, USA) using a method previously described.[11] Briefly, prior to 50 ng/mL flagellin or PBS pretreatment for 24 hours, HCECs were cultured in Keratinocyte Basal Medium (Lonza, Allendale, NJ, USA) overnight to starve cells from any growth factors. Then HCECs were challenged by *C. albicans* strain SC5314 for 4 hours. For detection, heat-killed *C. albicans* induced ISG15 expression and secretion in a type 1 IFN-dependent manner in cultured HCECs. Human primary (P3) CECs cultured in six-well plates were challenged with heat-killed *C. albicans* or CXCL10 with or without B18R protein (Thermo Fisher Scientific, Grand Island, NY, USA; a type 1 IFN receptor encoded by the B18R gene for neutralization of mouse and human IFNs). At 6 hours posttreatment, culture media were collected and trichloroacetic acid (TCA) precipitated; pellets were resuspended in SDS sample buffer and subjected to Western blotting with anti-human ISG15 antibody. Cells were also lysed and protein concentration determined. For each sample, 30-μg protein was used and probed with ISG15 antibody with β-actin as the loading control.

Clinical Scoring

For the assessment of clinical scores, the mice were color-coded and examined by two independent observers daily, one with the knowledge of codes and the other in a blind fashion, and photographed at 1, 3, 5, or 7 days after infection (dpi). If there was a conflict in clinical scoring, a third person, usually the corresponding author, would perform scoring from photographs. Ocular disease was graded in clinical scores ranging from 0 to 12, according to the scoring system developed by Wu et al.[39] A grade of 0 to 4 was assigned to each of the following three criteria—area of opacity, density of opacity, and surface irregularity—resulting in a maximum total score of 12. At the indicative time points, all infected corneas were photographed with a slide lamp or a dissection microscope to illustrate the disease progression.

Fungal Load Determination, Cytokine ELISA, and Myeloperoxidase (MPO) Measurement

We used our previously modified methods that allowed all three assays (fungal load, MPO determination, and cytokine ELISA measurement) to be performed with a single mouse cornea. Briefly, the corneas were excised from the enucleated

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Table. Mouse Primer Sequences Used for PCR

β-actin	GACGGCCAGGTCATCACTATTG	AGGAAGGCTGGAAAAGAGCC
IL-1β	AAGGAGAACCAAGCAACGACAAAA	TGGGGAACTCTGCAGACTCAAACT
IL1RA	GGGACCCTACAGTCACCTAA	GGTCCTTGTAAGTACCCAGAC
CXCL10	ATGAACCCAAGTGCTGCCGTC	TTAAGGAGCCCTTTTAGACCTTT
CRAMP	CTGTGGCGGTCACTATCAC	TGGGGCTCAGGATCCAGGTC
ISG15	TGCCTGCAGTTCTGTACCAC	AGTGCTCCAGGACGGTCTTA

eyes, minced, and homogenized in 100 μL PBS with Dounce Micro Tissue Grinder. The homogenates were divided into two. The first part was subjected to plate fungal counting. Aliquots (50 μL) of serial dilutions were plated onto YPD agar plates in triplicates. The plates were incubated 3 days at 25°C and fungal colonies were counted. The results were expressed as the mean number of CFU/cornea ± standard error of the mean. The second part of the homogenates was mixed with 5 μL 1% SDS and 10% Triton-X 100. For MPO assay, 30 μL homogenates was mixed with 270 μL hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). The samples were then subjected to three freeze-thaw cycles, followed by centrifugation at 16,000*g* for 20 minutes. Twenty microliters of the supernatant was mixed with 180 μL 50 mM phosphate buffer (pH 6.0) containing 16.7 mg/mL *O*,*O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide at a 1:30 ratio in a 96-well plate. The change in absorbance at 460 nm was monitored continuously for 5 minutes in a Synergy2 Microplate reader (BioTek, Winooski, VT, USA). The results were expressed in units of MPO activity/cornea. One unit of MPO activity corresponded to approximately 2.0×10^5 polymorphonuclear leukocytes (PMN). For ELISA, protein concentration was first determined and using Micro BCA protein assay kit (Pierce, Grand Island, NY, USA) and 5 μg total protein was used for ELISA assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Real-Time PCR

Mouse cornea RNA was extracted using RNeasy Mini Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. cDNA was generated with an oligo(dT) primer (Invitrogen, Grand Island, NY, USA). Generated cDNA was amplified by real-time PCR with the Power SYBR Green PCR Master Mix (AB Applied Biosystems, University Park, IL, USA) by using primers for mouse ISG15 and ISGylation associated genes (UBE1L, UBCH8, and HERC5), using β-actin as the reference gene (Table).

Western Blotting

The lysate of mouse cornea was examined by Western blotting using the method previously described.[13] The membranes were incubated with primary antibody rabbit anti-mouse ISG15 (R&D Systems; 1:100) overnight, then incubated with HRP conjugated second antibody goat anti-rabbit IgG (1:10,000) for 1 hour.

Immunohistochemistry

At the indicative time points, the corneas were excised from killed mice and were frozen in optimal cutting temperature (OCT) compound; posterior parts along with the lens were removed shortly after the global parts frozen. The corneas were cut into 6-μm thick sections by cryostat sectioning, and the sections were mounted to polylysine-coated glass slides. After a 10-minute fixation in 4% paraformaldehyde, the sections were blocked with 10 mM PBS, containing 2% BSA, for 1 hour at room temperature. Sections were then incubated with primary antibodies: rabbit anti-mouse ISG15 (BD, Franklin Lakes, NJ, USA; 1:100), rat anti-mouse NIMP-1 (BD; 1:1000), followed by the secondary antibody, Cy3 conjugated goat anti-rabbit IgG (Jackson Immunoresearch, Inc., West Grove, PA, USA), with or without FITC-labeled anti-rat NK1.1 (Jackson Immunoresearch, Inc.). Slides were mounted with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI). Controls were treated similarly, but the primary antibody was replaced with nonspecific rabbit IgG.

ISG15 siRNA Knockdown

Mice (five per group) were anesthetized as described. B6 mice corneas were first subconjunctivally injected with 10 μM ISG15 siRNA (ON-TARGETplus mouse ISG15 siRNA; GE Lifesciences, Pittsburgh, PA, USA) or control siRNA. After 4 hours, mice were pretreated with flagellin (500 ng in 5 μL PBS) or PBS (control), then these corneas were inoculated with *C. albicans* using the method described above. Eyes were examined to monitor the disease progression with microscope equipped with a digital camera at 24 hours postinfection (hpi).

The Application of Exogenous ISG15

For ISG15 pretreatment, mice (five per group) were anesthetized as described, and B6 mice corneas were first subconjunctivally injected with 2 μL recombinant ISG15 (CiruLex, Des Plaines, IL, USA), using BSA as control. After 4 hours, the corneas were inoculated with *C. albicans* using the method described above. Eyes were examined to monitor the disease progression with microscope equipped with a digital camera at 24 hpi. The fungal load, MPO activity, and Western blotting were performed as described above.

Statistical Analysis

Data were presented as the mean ± SD. Statistical differences among three or more groups were identified using 1-way ANOVA. Differences were considered statistically significant at $P < 0.05$. Between two groups, an unpaired, two-tailed Student's *t*-test was used to determine statistical significance for data from fungal counts, cytokine ELISA, and the MPO assay. A nonparametric Mann-Whitney *U* test was performed to determine statistical significance for clinical scores. Experiments were repeated at least twice to ensure reproducibility.

Results

C. albicans-Induced ISG15 Expression Was Enhanced by Flagellin Pretreatment in Cultured HCECs

Our genome-wide cDNA array of mouse CECs revealed that bacterial infection induced a moderate increase (1.743-fold) in ISG15 expression and flagellin pretreatment greatly augmented this upregulation (14.7154-fold increase over the control or 8.44-fold increase over the infected corneas).[9] To demonstrate

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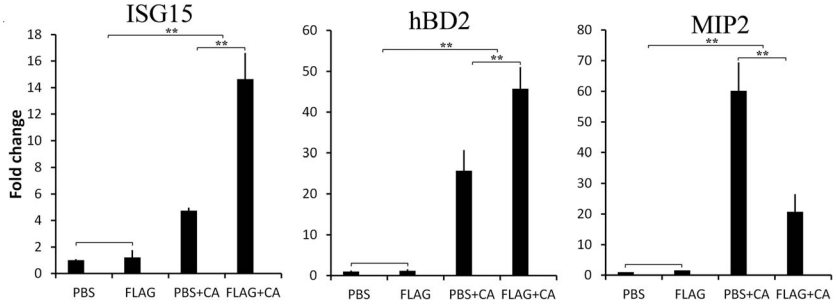

Figure 1. Heat-killed *C. albicans* induced and flagellin pretreatment further enhanced the expression of ISG15 in cultured HCECs. Normal or flagellin-pretreated (50 ng/mL, 24 hours) cells were challenged with heat-killed *C. albicans* for 4 hours and mRNA expression alterations of ISG15, AMP β-defensin 2, and proinflammatory cytokine MIP2 were quantified by comparing RNA fold changes of normal, uninfected cells versus *C. albicans*-infected and/or flagellin-pretreated cells using the real-time PCR. *P* values were generated using unpaired Student's *t*-test (**$P < 0.01$). The figure is a representative of four independent experiments.

that this expression pattern is not a species-specific event, we performed an in vitro assay using cultured human CECs (Fig. 1). Low dosage (50 ng/mL) of flagellin incubated for 24 hours had no effect on the expression of β-defensin-2, CXCL2, and ISG15. Challenge of cultured CECs with *C. albicans* resulted in the induction of ISG15, CXCL2, and β-defensin 2 (hBD2) at 4 hours post *C. albicans* challenge. In flagellin-pretreated cells, as shown previously, hBD2 expression was up while CXCL2 was downregulated. Flagellin pretreatment further enhanced the expression of ISG15 from 4.7-fold increase to 14.6-fold increase in *C. albicans* challenged HCECs.

Flagellin Pretreatment Augmented ISG15 Expression and ISG15 Conjugation in B6 Mouse Cornea During *C. albicans* Infection

Having confirmed ISG15 expression pattern in HCECs in vitro, we next investigated ISG15 expression pattern in vivo in B6 mouse corneas infected with *C. albicans*. In line with our previous studies, we used female mice in this study. Since ISGylation involves three enzymes, UBE1L, UbcH8, HERC5, we also assessed their expression along with ISG15 in mouse CECs using qPCR. Flagellin treatment alone for 24 hours did not significantly increase the expression of ISG15 or its associated enzymes. *C. albicans* clearly resulted in elevated expression of ISG15 (3.9-fold), UBE1L (3.2-fold), UBCH8 (2.6-fold), and HERC5 (4.0-fold) at 6 hpi (Figs. 2A–D). Flagellin pretreatment further augmented the expression of ISG15 (7.9-fold), UBE1L (4.6-fold), UBCH8 (3.7-fold), and HERC5 (5.0-fold) in *C. albicans* infected mouse CECs (Figs. 2A–D).

As antiviral activity of ISG15 is related to the ISGylation of host and viral proteins,[14,40,41] we next determined if ISG15 conjugation occurs in mouse CECs in response to *C. albicans* infection with or without flagellin pretreatment by Western blotting with rabbit anti-mouse ISG15 antibody. Low levels of ISG15 or ISG15 conjugates were detected in the control or flagellin pretreated mouse CECs. *C. albicans* infection resulted in enhanced ISG15 expression and ISG15 conjugation (Fig. 2E).

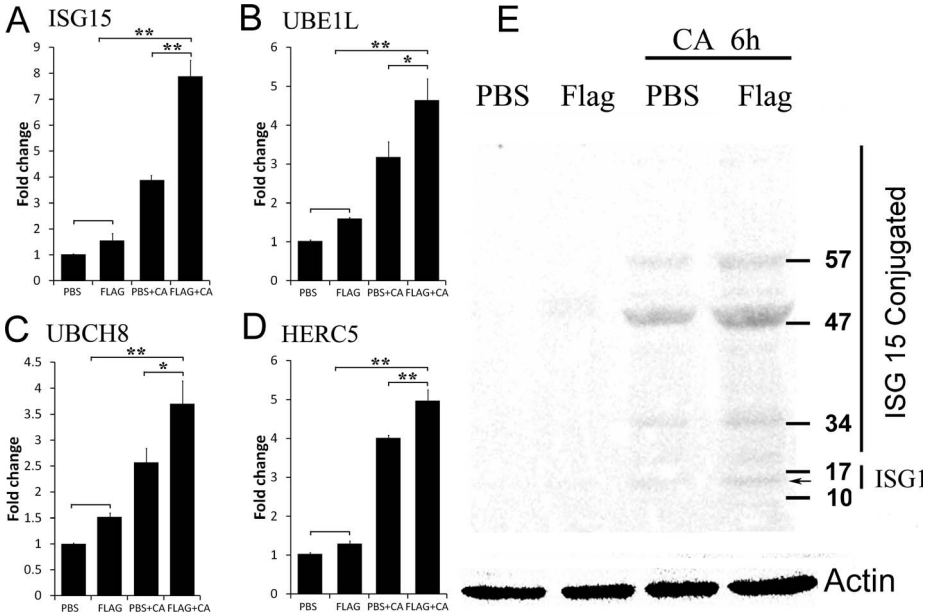

Figure 2. Fungal infection induced ISG15 and ISG15 conjugation were augmented by flagellin pretreatment in CECs in B6 mice. B6 mouse corneas were needle-scratched and topically applied with 5 μL flagellin (500 ng) or PBS and after 24 hours re-scratched and inoculated with 1×10^5 CFU *C. albicans*. At 6 hpi, CECs from flagellin pretreated and/or *C. albicans* infected mice were harvested for expression profile. Real-time PCR was performed to examine the expression of ISG15 (**A**), UBE1L (**B**), UBCH8 (**C**), and HERC5 (**D**). Western blotting was performed to examine the expression of ISG15 (*arrow*) and ISG15-conjugates (**E**). Data were pooled from three independent experiments ($N = 5$) and indicated *P* values were generated using unpaired Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

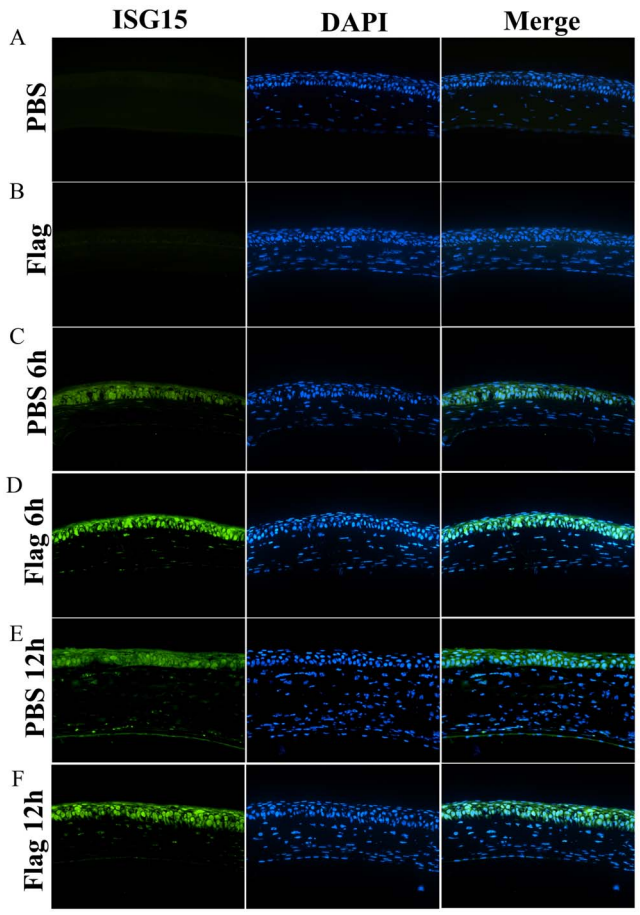

FIGURE 3. Distribution of ISG15 in *C. albicans* challenged corneas with or without flagellin pretreatment. B6 mouse corneas were pretreated and infected as described in Figure 2. At 0 (no fungal inoculation), 6, and 12 hpi, corneas were collected and embedded in OCT, followed by sectioning and immunostaining with rabbit anti-mouse ISG15 antibody (1:100). The corneas of the control (PBS), flagellin pretreated (**B**), *C. albicans* infected 6 hours (**C**), and 12 hours, Flag + *C. albicans* 6 hours (**E**), and Flag +*C. albicans* 12 hours (**F**) were examined under an epifluorescence microscope with DAPI staining for nuclei.

Both ISG15 expression and ISGylation were further augmented by flagellin pretreatment (Fig. 2E). However, the pattern of ISGylated proteins detected by Western blotting appeared to be the same in these samples.

ISG15 Is Expressed in *C. albicans* Infected Corneas With or Without Flagellin Pretreatment

To assess the expression of ISG15 at tissue levels, immunohistochemistry was performed in normal control and *C. albicans*-infected corneas at 6 hpi and 12 hpi with or without flagellin pretreatment. There was minimal ISG15 staining in noninfected corneas (Figs. 3A, 3B). Consistent with real-time PCR and Western blotting results, infection induced ISG15 expression; at 6 hpi, the entire epithelium was ISG15-positive with a few ISG15-positive infiltrated cells at anterior side of the stroma (Fig. 3C). The intensity of ISG15 staining in the epithelial layer in flagellin-pretreated corneas at this time point was stronger than the control with more infiltrated cells that evenly distributed within the stroma (Fig. 3D). At 12 hpi, there was apparent edema in the infected cornea without flagellin pretreatment, and epithelium was ISG15-positive with significantly more stromal ISG15-positive cells (Fig. 3E). In flagellin pretreated corneas, ISG15 staining remained strong in both epithelial cells and stromal infiltrates (Fig. 3F).

ISG15 Knockdown Increases Corneal Susceptibility to *C. albicans* and Abolishes Flagellin-Induced Protection in B6 Mouse Cornea

Having demonstrated that ISG15 was upregulated in *C. albicans*-challenged cultured HCECs and in B6 mouse CECs, we next investigated the role of ISG15 in corneal innate defense against *C. albicans* infection using a siRNA approach. As shown in Figure 4, the corneas treated with the control siRNA were partially covered with opacification at 1 dpi, while flagellin pretreatment protected the B6 mouse corneas from *C. albicans* infection (Fig. 4A). The cornea treated with ISG15 siRNA had much more severe keratitis with the whole corneas covered with opacification with or without flagellin pretreatment. In PBS pretreated corneas, 4.1×10^3 CFU *C. albicans* were recovered in control siRNA injected cornea, while ISG15 knockdown resulted in an increase of fungal loads (3.8-fold; Fig. 4B). While no fungi were detected in control siRNA injected, flagellin-pretreated corneas, ISG15 knockdown resulted in 9.9×10^3 CFU *C. albicans*. The increased severities of keratitis in ISG15-knockdown corneas were also apparent in terms of MPO activity (Fig. 4C) and CXCL2 expression (Fig. 4D).

The expression and ISGylation genes and ISG15 conjugation were also examined in B6 mice after ISG15 knockdown. At 24 hpi, whole corneas were collected and assayed by qPCR and Western blotting. qPCR results indicated ISG15 was silenced by siRNA in mouse corneas (Fig. 5A). Compared with the control, ISG15 knockdown significantly downregulated the expression of UBE1L (3.1-fold), UBCH8 (3.4-fold), and HERC5 (1.9-fold) in the control group, while in Isg15 knockdown group, all were lower than the controls (value 1; Fig. 5A). Western blotting of whole corneal samples revealed a decrease in ISG15 staining of free as well as conjugated proteins in *C. albicans* infected, ISG15 siRNA treated corneas with or without flagellin pretreatment (Fig. 5B).

ISG15 Knockdown Disturbs *C. albicans* Induced and Flagellin-Augmented IFN-γ Expression

ISG15 has been found to act as an extracellular cytokine to promote IFN-γ production and play an importance role in controlling human mycobacterial disease.[26] To determine if the levels of ISG15 in *C. albicans* infected corneas also influence the expression of IFN-γ, we assessed the expression of IFN-γ, in comparison with the expression of proinflammatory cytokine CXCL2 using qPCR (Fig. 5C). Since no detectable IFN-γ mRNA was found in the naïve B6 mouse corneas, we used *C. albicans*-induced expression without any treatment as the reference for its relative expression. Downregulation of ISG15 significantly dampened the infection-induced IFN-γ expression (5.6-fold decrease). Flagellin pretreatment markedly augmented *C. albicans*-induced expression of IFN-γ in CECs, and this upregulation was abolished in ISG15 siRNA treated corneas (45.8-fold decrease). The expression of CXCL2, on the other hand, was induced by *C. albicans* infection using the naïve corneas as a reference (value 1), and downregulation of ISG15 further increased its expression (2.7-fold increase). Flagellin pretreatment, as expected, dampened infection-induced CXCL2 expression; ISG15 downregulation reversed the effect of flagellin on CXCL2 expression (10.2-fold increase).

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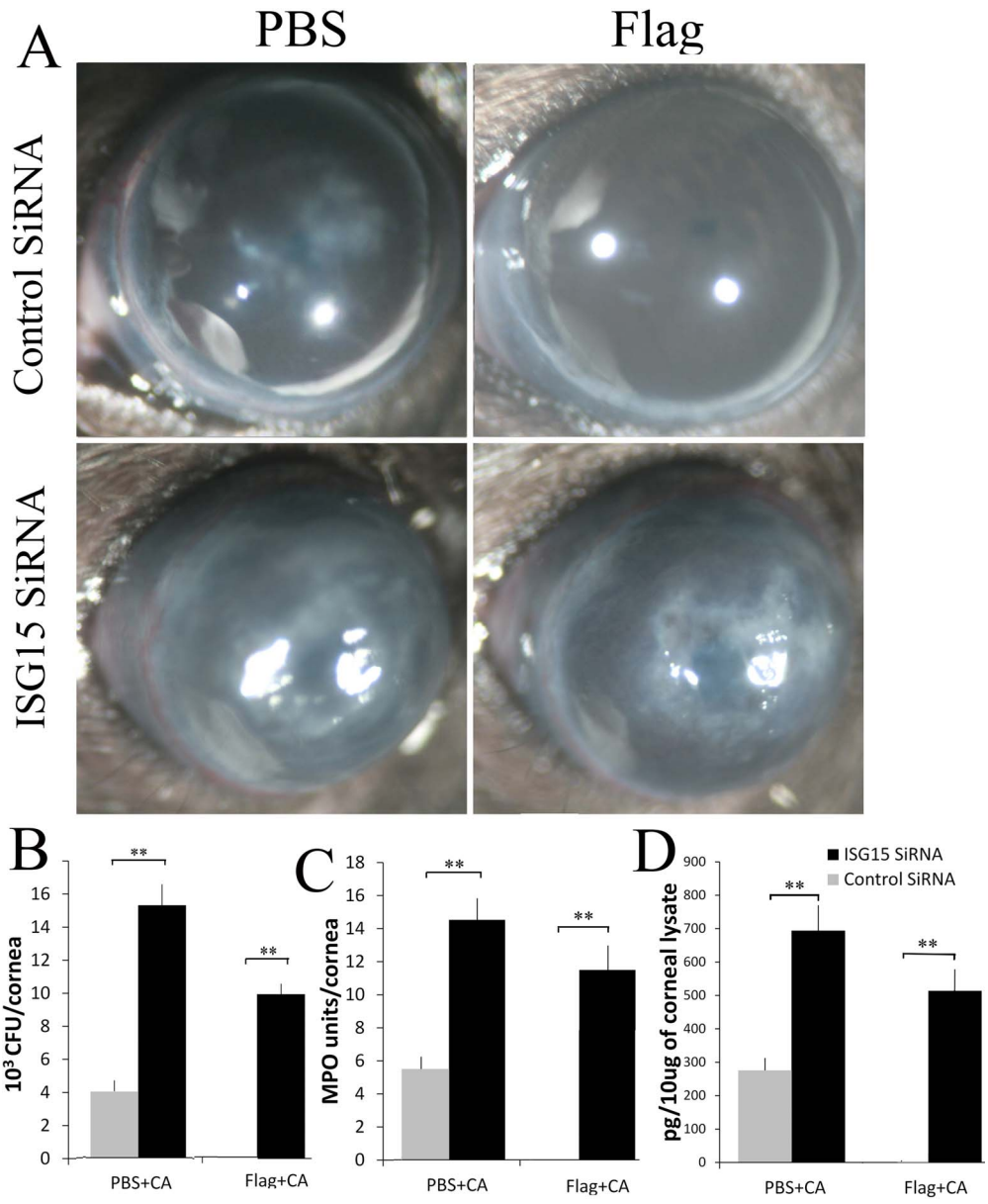

Figure 4. Knockdown ISG15 attenuated flagellin-induced protection against *C. albicans* infection in B6 mice. ISG15 SiRNA (5 μL, 10 μM) or control SiRNA was subconjectivally injected 6 hours before flagellin or PBS pretreatment. Then B6 mouse corneas were treated using the method described in Figure 2. At 24 hpi, eyes were examined to monitor the disease progression with a microscope equipped with a digital camera (**A**). The mouse corneas were then excised and homogenized for viable fungal load (**B**), MPO activity assay (**C**), and CXCL2 ELISA (**D**). Data were represented of three independent experiments ($N = 6$ each), and indicated P values were generated using unpaired Student's t-test ($^*P < 0.05$, $^{**}P < 0.01$).

Exogenous ISG15 Promotes Innate Defense Against *C. albicans* Infection in the Corneas

ISG15 may function extracellularly as an immunomodulator or intracellularly either as a free molecule or as a key enzyme for ISGylation. We next assessed the effects of exogenous ISG15, which was shown to function in a cytokine-like fashion.[17,27] As shown in Figure 6, subconjunctival injection of recombinant ISG15 resulted in much reduced severity of *C. albicans* (Fig. 6A), including decreased fungal burden (Fig. 6B) and PMN infiltration (Fig. 6C).

To determine how exogenous ISG15 might affect innate immune response to *C. albicans* infection, we assessed the expression of cytokines, chemokines, and innate defense molecules, using qPCR in the whole corneas collected at 24 hpi (Fig. 6D). Presence of recombinant ISG15 alone had no effect on the expression of proinflammatory cytokines IL-1β and CXCL2, but induced marked upregulation of IL-1RaV2 (encoding secreted form), CXCL10, and CRAMP. While the presence of ISG15 exhibited no effect on the expression of IL-1Ra, CXCL2, CXCL10, and CRAMP, it significantly suppressed the expression of IL-1β in *C. albicans*-infected CECs.

To determine if IFN-γ expression was mediated by ISG15 in normal and infected corneas, we performed ELISA and observed while ISG15 alone had no effect on IFN-γ expression; infection-induced expression at the protein levels was regressed by ISG15 in B6 mouse cornea (Fig. 6E).

We next investigated HCECs secreted ISG15 in response to heat-killed *C. albicans* challenge in a type 1 IFN receptor-dependent manner. Because infection or flagellin-pretreatment does not alter the pattern of ISGylation in vivo, we next

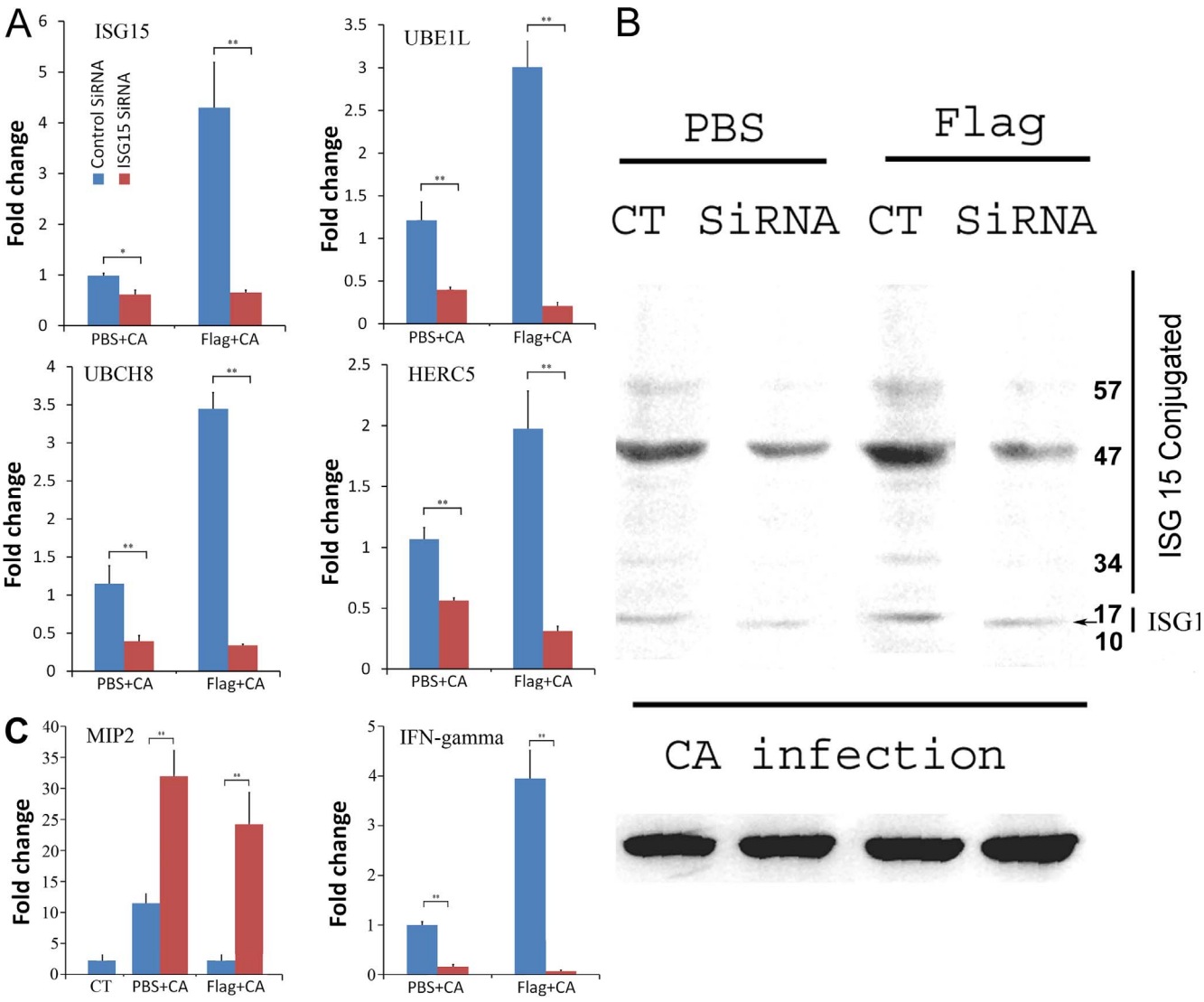

Figure 5. ISG15 knockdown affects ISGylation and IFN-γ expression of *C. albicans* infection in *C. albicans*-infected B6 mouse corneas. ISG15 and the control siRNA treated and *C. albicans*-infected mouse corneas, described in Figure 4, were collected at 1 dpi and processed for qPCR or Western blotting with ISG15 antibody. qPCR results were presented as fold increase over the control siRNA treated *C. albicans*-infected mouse corneas (value 1; **A**). Western blotting was performed to examine the expression of ISG15 (*arrow*) and ISG15-conjugates (**B**). For MIP2 and IFN-γ expression, noninfected corneas were used as the control for MIP2, while IFN-γ was undetectable. Data were pooled from three independent experiments ($N = 3$), and indicated P values were generated using unpaired Student's t-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

investigated whether ISG15 was secreted and how this secretion was regulated using cultured human primary CECs (Fig. 7). CECs cultured in KBM expressed and secreted basal detectable levels of ISG15 with or without B18R, a type 1 IFN-neutralizing protein. Challenging the cultured cells with heat-killed *C. albicans* markedly augmented the levels of free ISG15 and secreted ISG15 in type 1 IFN-dependent manner, while the levels of conjugated forms were elevated with or without B18R.

Discussion

Using flagellin as an immunostimulant to activate innate immunity and *C. albicans* as a model pathogen, we report in the current study that ISG15 was induced in response to *C. albicans* infection, and this elevated expression was further augmented by flagellin pretreatment in vitro in cultured human CECs and in vivo in B6 mouse corneas. At the tissue level, ISG15 was found mostly in epithelium along with some ISG15-positive infiltrated cells. The downregulation of ISG15 by siRNA greatly increased the severity of *C. albicans* keratitis and abolished flagellin-induced protection. Importantly, exogenous ISG15 enhanced innate immunity and prevented the corneas from *C. albicans* infection. These protective effects may be related to the induced expression of anti-inflammatory factor IL-1Ra, the multifunctional chemokine CXCL10, and cathelicidin antimicrobial peptide, as their expression was induced by exogenous ISG15 in B6 mouse corneas. Since *C. albicans* is an extracellular pathogen and since infection did not alter ISGylation pattern in vitro and in vivo, we suggested that ISG15 exerted its protective function as an extracellular immunomodulatory.

ISG15 is mostly a type 1 IFN-induced gene.[42] Given the IFN-dependent expression of the ISG15 gene, physiological and pathologic studies of ISG15 and ISGylation have been mostly focused on antiviral activity.[41,43–45] In addition to various viruses, bacterial infection has also been shown to induce the

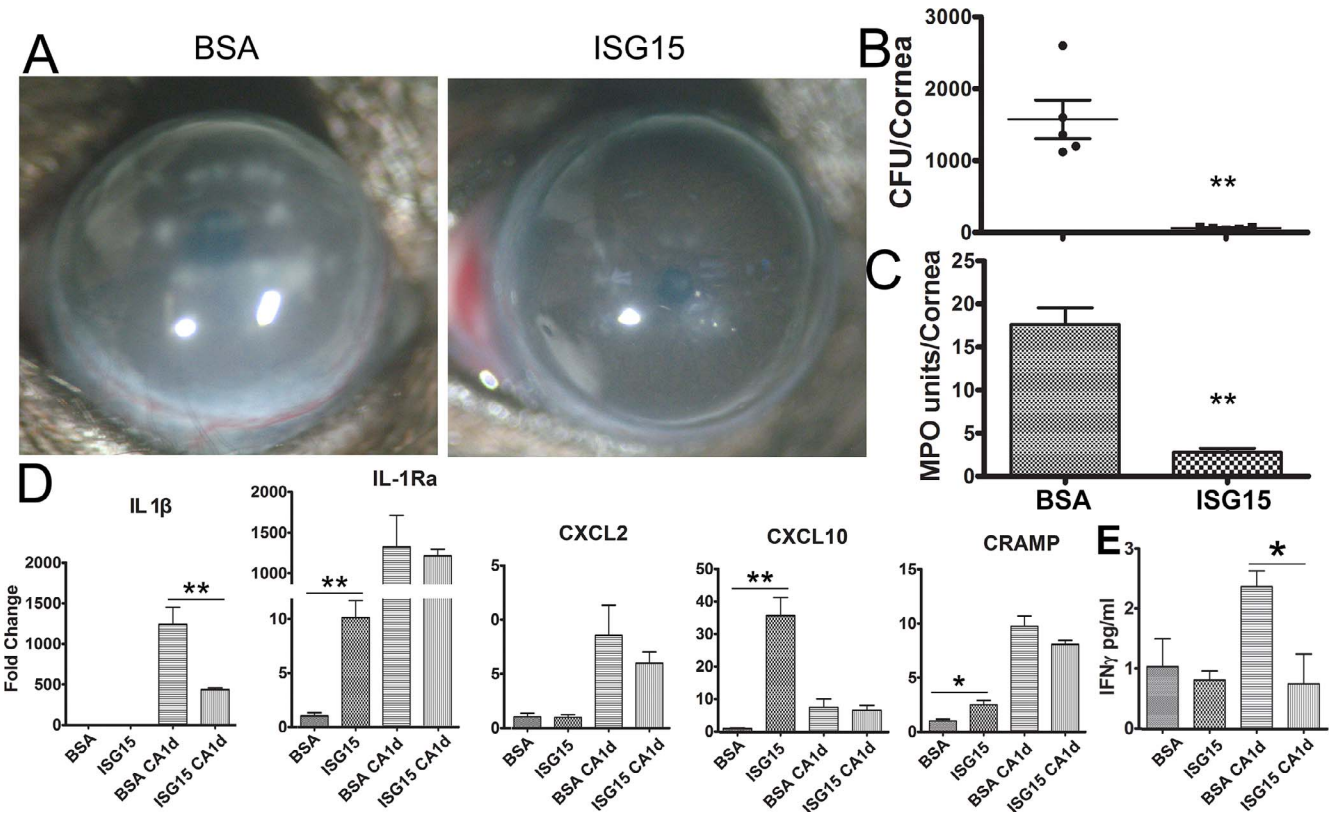

Figure 6. Recombinant ISG15 reduces the severity of *C. albicans* keratitis and affects the expression of innate immune mediators in B6 mouse corneas. Mice were subconjunctivally injected with either recombinant human ISG15 (200 ng in 5 μL 0.1% BSA) or 0.1% BSA as control. Mouse corneas were inoculated with *C. albicans* 4 hours after the injection of recombinant ISG15. (**A**) The infected corneas were photographed daily. (**B**) At 1 hpi, the corneas were excised and subjected to fungal load (**B**) and MPO unit determination (**C**). *P* values were generated by using Mann-Whitney *U* test (**B**) or unpaired Student's *t*-test (**C**, **D**). $^{*}P < 0.05$ and $^{**}P < 0.01$. Data are representative of three independent experiments with five mice per group (**B**: median + interquartile range; **C**, **D**: mean + SEM). To determine the effects of ISG15 on corneal gene expression, recombinant ISG15-treated corneas with or without *C. albicans* infection were collected and subjected to qPCR analysis (**D**) and ELISA for IFN-γ. Data were pooled from three independent experiments ($N = 3$), and indicated *P* values were generated using unpaired Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

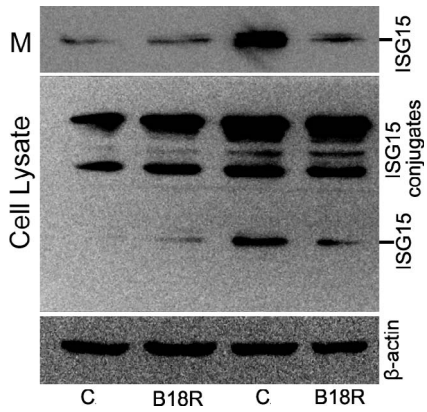

Figure 7. Heat-killed *C. albicans* induced ISG15 expression and secretion in a type 1 IFN-dependent manner in cultured HCECs. Human primary (P3) CECs cultured in six-well plates were challenged with heat-killed *C. albicans* or CXCL10 with or without B18R protein (a type 1 IFN receptor encoded by the B18R gene for neutralization of mouse and human IFNs). At 6 hours posttreatment, culture media were collected and TCA precipitated; pellets were resuspended in SDS sample buffer and subjected to Western blotting with anti-human ISG15 antibody. Cells were also lysed and protein concentration determined. For each sample, 30-μg protein was used and probed with ISG15 antibody with β-actin as the loading control. *Bold line*: free ISG15. The figure is a representative of three samples for each condition.

expression of ISG15.[46] Most reports in the literature demonstrated the expression of ISG15 in immune cells. To date, there was no study reporting the expression of ISG15 in the cornea or in any tissues in response to fungal infection. Our genome-wide cDNA array using isolated CECs of *P. aeruginosa* infected corneas during initial hours of infection, 6 hpi, showed infection-induced and flagellin-enhanced upregulation of ISG15 mRNA in B6 mouse corneas. In this study, we reported that fungal infection also induced the expression of ISG15, which was further enhanced by flagellin pretreatment in B6 mouse cornea, mostly in the epithelium. We also showed that other enzymes involved in ISGylation were upregulated in *C. albicans*-infected corneas. This, however, may be simply related to the fact that these three enzymes were also regulated by type 1 IFNs.[18] Hence, fungal infection-induced and flagellin-enhanced expression of ISG15 may be a component of innate defense machinery against fungal infection.

Recently, ISG15 was found to be the causative gene for Mendelian susceptibility to mycobacterial disease, a rare condition characterized by predisposition to clinical disease caused by weakly virulent mycobacteria, such as BCG vaccines and environmental mycobacteria, in otherwise healthy individuals, none of which displayed higher than usual susceptibility to viral infection.[27,47,48] In addition to mycobacterial infection, ISG15 expression has also been shown to restrict Listeria infection in vitro and in vivo in a type 1 IFN-independent manner and a cytosolic surveillance pathway-dependent manner. The STING senses aberrant DNA species or cyclic

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dinucleotides in the cytosol of the cell and signals through TBK1, IRF3, and IRF7.[49] Like viruses, mycobacterial and Listeria are intercellular pathogens and detected mostly by intracellular sensors, such as TLR3, 7, and STING.[50] *C. albicans*, on the other hand, is an extracellular pathogen, whose recognition at least at the early stage of infection is through cell surface receptors, such as TLR2 and 4. The rapid induction of ISG15 in response to *C. albicans* suggests a TLR-regulated response in the cornea.

To determine if ISG15 plays a role in innate immunity against fungal keratitis, we used siRNA-knockdown and recombinant proteins. Knockdown of ISG15 increased the severity of fungal keratitis and abolished flagellin-induced protection. Strikingly, in ISG15 knockdown corneas, there were marked increases in fungal burden, PMN infiltration, and expression of CXCL2 at the protein level. Hence, this study is expanding the role of ISG15 in modulating innate immunity to fungal infection.

To date, ISG15, when it is expressed, has been found intracellularly as a free molecule, in the conjugated form, or extracellularly in a free form. For antiviral function, ISG15 can catalyze ISGylation of host and viral proteins, the latter may prevent viral replication and/or entry/uncoating from the infected cells.[33,51] The loss of ISG15 increased the susceptibility to Salmonella infection in ISG15-deficient mice, while deficiency in USP18, a de-conjugating protease of ISG15, restricted the growth of Salmonella typhimurium more efficiently than wild-type mice.[24] These data appear to suggest that ISGylation is required for bacterial clearance in the mice. However, inherited ISG15 deficiency is associated with severe mycobacterial disease in both mice and humans. This infectious phenotype probably results from the lack of secreted ISG15, because patients and mice with other inborn errors of IFN-γ immunity also display mycobacterial diseases.[27] Since *C. albicans* is an extracellular pathogen, it is plausible that extracellular ISG15 might be involved in innate immune response to *C. albicans*. Indeed, our in vitro study using cultured human CECs demonstrated the upregulation of expression and robust secretion of ISG15 in response to *C. albicans* in a type 1 IFN-dependent manner. As seen in vivo, heat-killed *C. albicans* exhibited no detectable influence on the pattern of ISGylation. Moreover, recombinant ISG15 markedly induced the expression of CXCL10 and greatly reduced the severity of *C. albicans* keratitis, including eradication of invading pathogens, and greatly reduced infiltration of PMN suggests that ISG15 functions as an immunomodulator of innate immunity against keratitis causing pathogens.[10,11]

The mechanism underlying ISG15 secretion is unknown.[17] A recently study found ISG15 in the exosomes of TLR3-activated human brain microvascular endothelial cells, which are able to transport the antiviral molecules to macrophages.[52] It is plausible that human CEC-produced ISG15 may also be delivered to infiltrated immune cells, such as natural killer (NK) cells that were shown to play a critical role in innate defense of microbial keratitis.[11] In *C. albicans*-infected corneas, NK cells organized to form a large cluster at a lesion site, and depletion of the NK cells resulted in severe *C. albicans* keratitis and eventual perforation in otherwise self-healing B6 mouse corneas in an IFNγ-related manner.[11] In this study, we reported that ISG15 silencing resulted in the total suppression of infection-induced and flagellin pretreatment-augmented IFN-γ expression in CECs at early time of infection (6 hpi). Pulmonary and oral epithelial cells were also shown as a source of IFN-γ in response to *Mycobacterium tuberculosis* and *C. albicans* infection, respectively.[53,54] Interestingly, exogenous ISG15 was shown to suppress the expression of IFN-γ at the protein levels, as well as IL-1β at the mRNA levels in *C. albicans*-infected corneas at 1 dpi. It is plausible that the lack of pathogen burden at this time point results in less innate inflammatory response. More importantly, ISG15 alone was able to induce the expression of anti-inflammatory cytokine IL-1Ra, multifunctional chemokine CXCL10, and antimicrobial gene cathelicidin.[55] Our previous studies revealed that those are important mediators and/or effectors of innate mucosal immunity against microbial infection of the corneas.[10,11,56] Hence, ISG15 functions as immunomodulator of fungal keratitis in the cornea by suppressing inflammation-downregulating IL-1β and IFN-γ, and inducing fungicidal molecules CXCL10[10] and cathelicidin.[57]

In summary, this study for the first time provides the direct evidence that ISG15 functions as an immunomodulator and plays a regulatory role in innate immunity against fungal infection in the cornea, and that manipulation of ISG15 may be used to enhance innate immunity and reduce tissue damage caused by infection and/or excessive inflammatory response.

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