ISG15 Acts as a Mediator of Innate Immune Response to *Pseudomonas aeruginosa* Infection in C57BL/6J Mouse Corneas

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Purpose. IFN-stimulated gene (ISG) 15 is a type 1 IFN-induced protein and known to modify target proteins in a manner similar to ubiquitylation (protein conjugation by ISG15 is termed *ISGylation*). We sought to determine the role of ISG15 and its underlying mechanisms in corneal innate immune defense against *Pseudomonas aeruginosa* keratitis.

Methods. ISG15 expression in cultured human corneal epithelial cells (HCECs) and mouse corneas was determined by PCR and Western blot analysis. Gene knockout mice were used to define the role of ISG15 signaling in controlling the severity of *P. aeruginosa* keratitis, which was assessed with photographing, clinical scoring, bacterial counting, myeloperoxidase assay, and quantitative PCR determination of cytokine expression. Integrin LFA-1 inhibitor was used to assess its involvement of ISG15 signaling in *P. aeruginosa*–infected corneas.

Results. Heat-killed *P. aeruginosa* induced ISG15 expression in cultured HCECs and accumulation in the conditioned media. *Isg15* deficiency accelerated keratitis progress, suppressed IFNγ and CXCL10, and promoted IL-1β while exhibiting no effects on IFNα expression. Moreover, exogenous ISG15 protected the corneas of wild-type mice from *P. aeruginosa* infection while markedly reducing the severity of *P. aeruginosa* keratitis in type 1 IFN-receptor knockout mice. Exogenous ISG15 increased bacteriostatic activity of B6 mouse corneal homogenates, and inhibition of LFA-1 exacerbated the severity of and abolished protective effects of ISG15 on *P. aeruginosa* keratitis.

Conclusions. Type 1 INF-induced ISG15 regulates the innate immune response and greatly reduces the susceptibility of B6 mouse corneas to *P. aeruginosa* infection in an LFA-1-dependent manner.

Keywords: ISG15, interferon signaling, bacterial keratitis, antimicrobial peptide

The avascular cornea has two specialized functions: forming a protective barrier and serving as the main refractive element of the visual system.[1] The limited immune surveillance combined with the hypersensitivity of transparency to inflammation-mediated damage of the cornea makes it crucial that ocular surface cells are able to quickly recognize and respond to microbial infection.[2,3] Under normal conditions, the cornea is remarkably resistant to infection. However, when the epithelial barrier is breached, which often occurs during routine contact lens wearing or when immune function is compromised such as that in diabetic patients, opportunistic pathogens such as *Pseudomonas aeruginosa* can gain access to the stratified epithelium and eventually to the stroma, causing infectious keratitis.[4–8] *P.* aeruginosa is an opportunist pathogen associated with bacterial keratitis, especially in extended-wear contact lens users.[9,10] If not treated promptly and properly, significant vision loss or even loss of the eye may occur.[11–13] As such, *P. aeruginosa* keratitis is still a major concern, especially at the dawn of a postantibiotic era.[14] Better understanding of how the ocular surface initiates innate immune response to invading pathogens is of importance for developing therapeutic strategies of adjunctive treatment of microbial keratitis.

Using genome-wide cDNA array of infected and flagellin-pretreated and infected corneas, we identified a large amount of differentially expressed genes associated with innate mucosal immune protection, many of which are interferon-stimulated genes (ISGs).[15] Among these genes, ISG15 is induced upon *P. aeruginosa* and greatly augmented by flagellin pretreatment, suggesting a protective role of the gene. ISG15 is a ubiquitin-like modifier that can bind covalently to a cellular and pathogenic protein, a process termed *ISGylation*. It is strongly induced by type I interferons (IFN-α/β) and by viral, bacterial, fungal, and parasite infections.[16–18] ISG15 is also known to be expressed in innate immune cells and residential epithelial cells.[19–21] In addition to ISGylation, ISG15 acts as an extracellular

cytokine/chemokine that induces the production of IFNγ from macrophages, epithelial cells, and NK cells; promotes NK cell activity; and increases antigen presentation and lysosome activity of macrophages.[22,23] Our previous study demonstrates that ISG15 plays a critical role in controlling fungal keratitis.[16] However, relatively little is known about the functional significance of ISG15 in the induction of innate immune responses to *P. aeruginosa* keratitis.

In this study, we explored the biological function of ISG15 and the mechanisms underlying corneal mucosal innate defense against *P. aeruginosa* strain ATCC 19660 (cytotoxic and noninvasive) and demonstrated that ISG15 was mostly synthesized and secreted from epithelial cells challenged by *P. aeruginosa*, especially at an early stage of infection. We report that the lack of *Isg15* increased severity of *P. aeruginosa* keratitis and altered the expression of many regulatory genes, including *Cxcl10* and *Ifn*γ. Our results indicate a key role of ISG15 in controlling *P. aeruginosa* infection and keratitis pathogenesis.

Methods

Animals

Wild-type (WT) C57BL6 (B6) mice (8 weeks of age; 20–24 g) and *Isg15* knockout mice (B6.129P2-Isg15tm1Kpk/J) breeding pairs were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Isg15* knockout mice were bred in-house and their pups were subjected to genotyping before use. Animals were treated in compliance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committee of Wayne State University approved all animal procedures.

Cell Culture of Primary Human Corneal Epithelial Cells

Primary human corneal epithelial cells (HCECs) were isolated from human donor corneas obtained from Eversight Michigan (Ann Arbor, MI, USA) using a previously described method.[24] P4 of the primary HCECs were used for the experiments. Confluent cultured cells were starved overnight in Keratinocyte Growth Medium-2 (KGM-2) (Lonza, Alpharetta, GA, USA). Subsequently, cells were challenged with heat-killed (H-K) *P. aeruginosa* (1:100 multiplicity of infection) for the indicated times.

Clinical Examination

The eyes were examined daily to monitor the disease progression with a dissection microscope equipped with a digital camera. For the assessment of clinical scores, mice were color coded and examined in a masked fashion by two independent observers at 1, 3, and 5 days postinfection (dpi) to visually grade the disease severity. Ocular disease was graded in clinical scores ranging from 0 to 12, according to the scoring system developed by Wu et al.[25] At 1 or 3 dpi, all infected corneas were photographed with a dissection microscope to illustrate the disease progression.

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

As previously described,[26] the corneas were excised from the enucleated eyes, minced, and homogenized in 100 µL PBS with a TissueLyser (Retch, Newtown, USA). The homogenates were divided into two samples. The first was subjected to the counting of bacteria colonies. Aliquots (100 µL) of serial dilutions were plated onto *Pseudomonas* isolation agar (BD Biosciences, San Jose, CA, USA) plates in triplicate. The plates were incubated overnight at 37°C, and the bacteria colonies were counted. The results were expressed as the mean number of CFU/cornea ± SEM. The second homogenate sample was mixed with 5 µL 1% SDS and 10% Triton X-100 with a 200-µL pipette. For the MPO assay, 30 µL homogenate 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 of 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 well of a 96-well plate. The change in absorbance at 460 nm was monitored continuously for 5 minutes with a microplate reader (Synergy2; 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.[27] Protein concentration was first determined using a protein assay kit (Micro BCA; Pierce, Rockford, IL, USA). One microgram of total protein was used to perform ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

RNA Extraction and Real-Time PCR

For RNA isolation, whole corneas or epithelial cells scraped off from corneas were frozen in liquid nitrogen immediately. RNA was extracted from the collected epithelial cells using RNeasy Mini Kit (QIAGEN, Germantown, MD, USA), according to the manufacturer's instructions. cDNA was generated with an oligo(dT) primer (Invitrogen, Carlsbad, CA, USA) followed by analysis using real-time PCR with the Power SYBR Green PCR Master Mix (AB Applied Biosystems, Foster City, CA, USA) based on expression of β-actin. The primer pairs are shown in the Table.

In Vitro Assay of Antibacterial Activity of Recombinant ISG15

Mice corneas were subconjuctivally injected with recombinant ISG15 or BSA for 6 hours, excised from the enucleated eyes, minced, and homogenized in 100 µL PBS with a TissueLyser (Retch). Then, 400 µL PBS with homogenized corneas were incubated with 100 CFU of *P. aeruginosa* at 37°C for 30 and 60 minutes. Serial dilutions of each reaction mixture were made to inoculate agar plates. Samples (100 µL) were spread evenly over the surface of the plates with sterile glass spreaders. After incubation at 37°C for overnight, the number of colonies was counted. Experiments were done at least twice.

Western Blot

Primary HCECs were lysed with RIPA buffer and centrifuged to obtain supernatant. Protein concentration was determined by BCA assay. The protein samples were separated by SDS-PAGE and electrically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes

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Table. Mouse Primer Sequences Used for PCR

Gene	Primer	Sequence (5′→3′)
β-actin	GACGGCCAGGTCATCACTATTG	AGGAAGGCTGGAAAAGAGCC
Il1b	AAGGAGAACCAAGCAACGACAAAA	TGGGGAACTCTGCAGACTCAAACT
Tslp	AGGCTACCCTGAAACTGAG	GGAGATTGCATGAAGGAATACC
Il-36α	CCAAGAACTGGGGGAAATCT	GGAGGGCTCAGCTTTCTTTT
Cxcl10	ATGAACCCAAGTGCTGCCGTC	TTAAGGAGCCCTTTTAGACCTTT
Ifnα	GCTCACCATTTCAACCAGT	GATGGTTTCAGCCTTTTGGA
Ifnβ	CATTACCTGAAGGCCAAGGA	CAGCATCTGCTGGTTGAAGA
Ifnγ	CAGAGCCAGATTATCTCTTTCTACCTCAGAC	CTTTTTCGCCTTGCTGTTGCTGAAG

were blocked with 3% BSA and subsequently incubated with ISG15 antibody (Cell Signaling Technology, Danvers, MA, USA) and s HRP (Horseradish Peroxidase)-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA). Signals were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Pittsburgh, PA, USA). β-Actin (Sigma-Aldrich, St. Louis, MO, USA) was used as the loading control.

Statistical Analyses

Data were presented as means ± SDs. Statistical differences among three or more groups were identified using 1-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

ISG15 Expression and Secretion Are Upregulated in Cultured HCECs in Response to Heat-Killed *P. aeruginosa*

Our genome-wide cDNA array study revealed that *Isg15* was upregulated in mouse corneal epithelial cells (CECs) in response to *P. aeruginosa* infection.[28] To validate its expression pattern and to ensure a similar event occurring in human cornea, we first assessed the expression of ISG15 in cultured HCECs challenged with H-K *P. aeruginosa* (Fig. 1). H-K *P. aeruginosa* has been used to study HCECs' response to the pathogen, mimicking the initial response of the cornea to infection.[29,30] Challenging the confluent culture of HCECs with 1:100 multiplicity of infection resulted in robust upregulation of ISG15 in both free and conjugated forms in HCECs (Fig. 1A). Importantly, unconjugated ISG15 was observed in the culture media of HCECs starting at 4 hours poststimulation (Fig. 1B), suggesting that ISG15 was upregulated and a portion of ISG15 was secreted in HCECs in response to *P. aeruginosa* challenge.

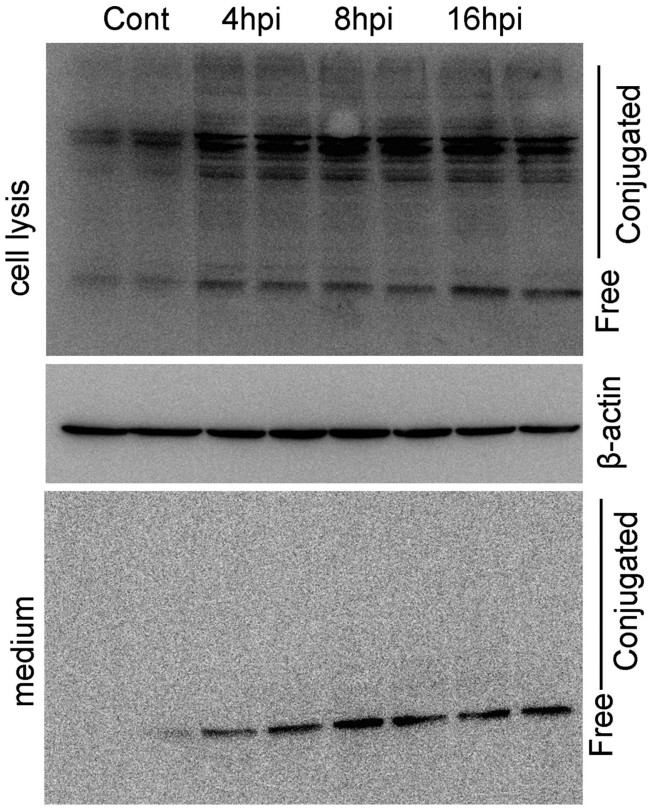

Figure 1. ISG15 expression in H-K ATCC-treated primary HCECs and conditioned media. Cultured HCECs were challenged with 100:1 H-K *P. aeruginosa* cells. Cells and conditioned media were collected at 4, 8, and 16 hours postchallenge and processed for ISG15 Western immunoblotting analysis. The results are representative of two independent experiments.

Isg15 Deficiency Worsens the Outcome of *P. aeruginosa* Keratitis

To further assess the role of ISG15 in *P. aeruginosa* keratitis, we used *Isg15*$^{-/-}$ mice, which had no phenotypic changes observed.[31] WT and *Isg15*$^{-/-}$ corneas were inoculated with 10,000 CFU *P. aeruginosa*; at 1 dpi, there was more opacification in *Isg15*$^{-/-}$ than in the WT mouse corneas. At 3 dpi, *Isg15* deficiency resulted in more severe keratitis with a significantly higher clinical score (6.2 ± 0.8 vs. 10.6 ± 0.8), bacterial burden (8.2×10^5 vs. 3.7×10^6 CFU), and MPO levels (96.6 vs. 212.1 units) than that of WT mice (Fig. 2).

We next investigated the expression of IFNs and other innate mediators in *Isg15*$^{-/-}$ mouse corneas in response to *P. aeruginosa* infection using quantitative PCR (qPCR) (Fig. 3). Among five genes on the list, only *Il36a* (encoding IL-36α) had greatly suppressed expression, compared to WT, in uninfected *Isg15*$^{-/-}$ mouse corneas. At 6 hpi (hours post infection), the expression of all five genes at three time points was significantly increased in response to *P. aeruginosa* infection in WT mice, with the value of naive, WT corneas set as 1 (WT). *Isg15* deficiency suppressed *Ifng* (encoding IFNγ), *Cxcl10*, and *Ii36a* (*encoding IL-36α*); augmented *Il1b* (encoding IL-1β); and exhibited no effect on *Ifna* (encoding IFNα) expression in *P. aeruginosa*–infected corneas. At 1 dpi, *Ifna* remained largely unchanged, and *Il1b* expression was markedly higher than that of 6 hpi

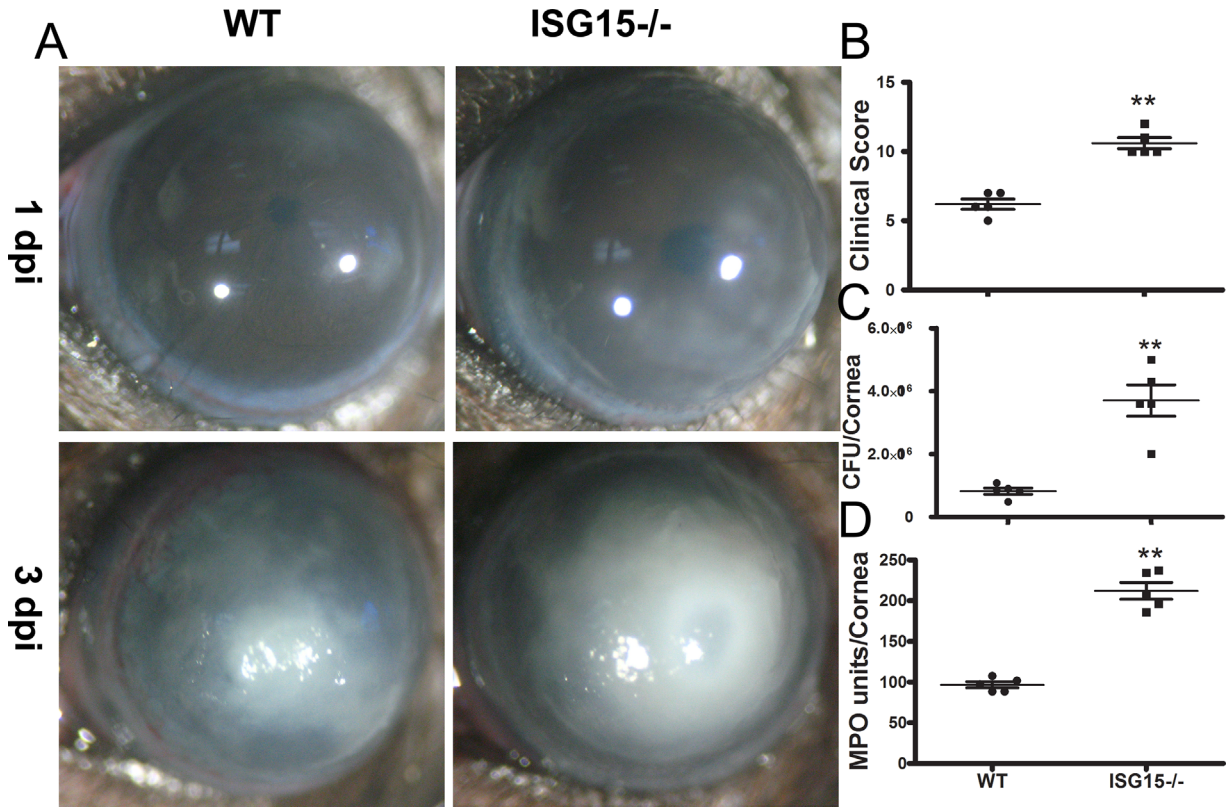

Figure 2. *Isg15* deficiency increased the severity of *P. aeruginosa* keratitis in B6 mice. WT or *Isg15*$^{-/-}$ B6 mouse corneas were scarified and inoculated with 1.0 × 10^4 CFU of *P. aeruginosa*. The infected corneas (n = 5) were photographed at 1 and 3 dpi (**A**). At 3 dpi, the corneas were clinically scored (**B**), excised, and subjected to bacterial counting (**C**) with the results presented as CFU *P. aeruginosa* per cornea and to MPO determination (units/cornea) (**D**). The results are representative of two independent experiments (n = 5 each), and P values were generated using 1-way ANOVA. **$P < 0.01$.

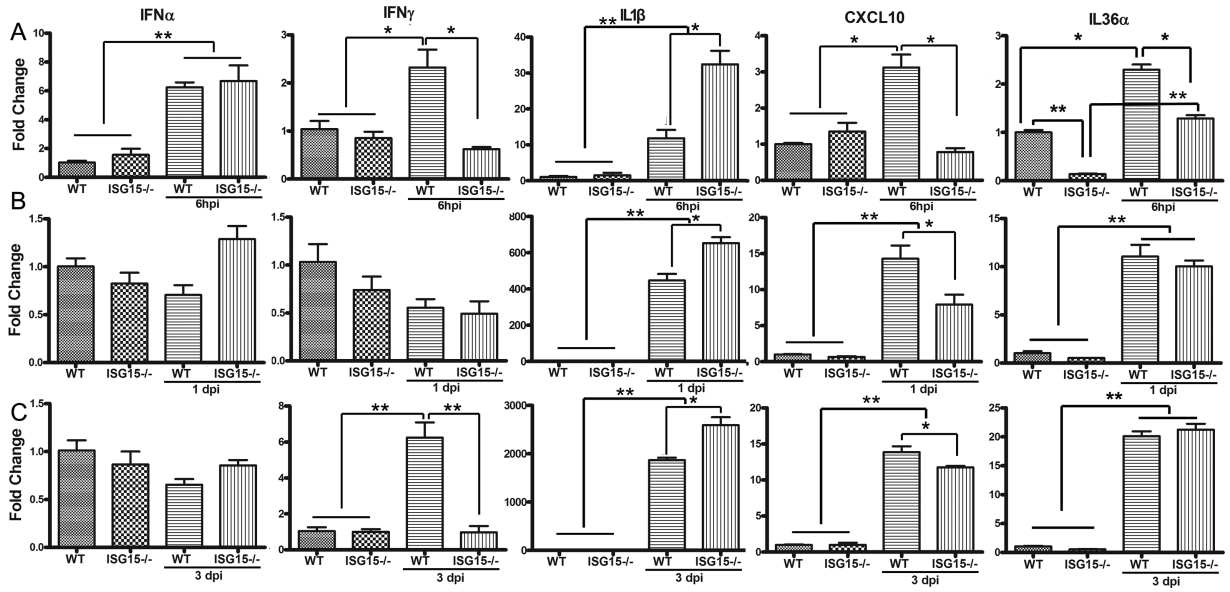

Figure 3. Effects of *Isg15* deficiency on the gene expression of B6 mouse corneas in response to *P. aeruginosa* infection. WT or *Isg15*−/− mouse corneas were inoculated with 1.0 × 10^4 CFU of *P. aeruginosa*. The scrapped epithelia (**A**) or whole corneas (**B**, **C**) were subjected to RNA isolation and real-time PCR analysis at 6 hpi (**A**), 24 hpi (**B**), and 3 dpi (**C**). The results are presented as the increase (fold) over the value for WT naive corneas (set at 1) after normalization to the level of β-actin as the internal control. The results are representative of two independent experiments, each with three corneas. *$P < 0.05$, **$P < 0.01$ (1-way ANOVA).

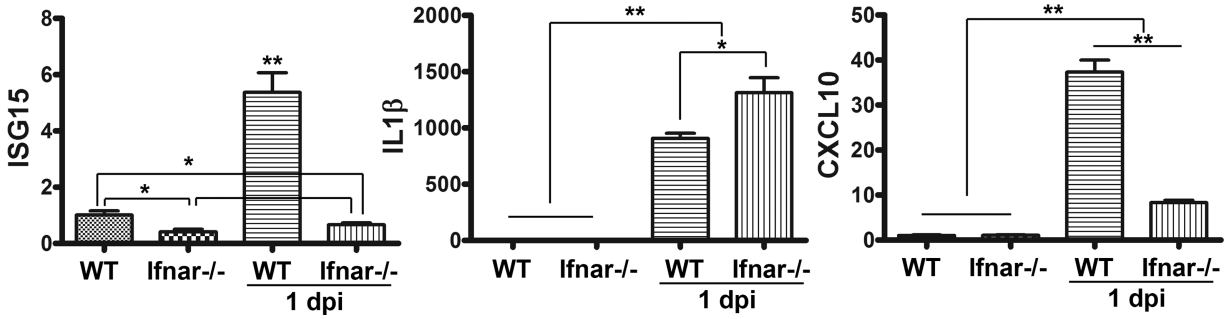

Figure 4. Effects of *Ifnar* deficiency on *Isg15* and *Cxcl10* expression in B6 mouse corneas in response to *P. aeruginosa* infection. WT or *Ifnar*$^{-/-}$ mouse corneas were scarified and inoculated with 1.0×10^4 CFU of *P. aeruginosa* at 0 hours. At 1 dpi, the infected corneas, along the corneas isolated from naive WT and *Ifnar*-deficient mice, were processed for qPCR analysis for the mRNA expressions of *Isg15*, *Il1b*, and *Cxcl10*. The results are presented as the increase (fold) over the value for wild-type naive corneas (set at 1) after normalization to the level of β-actin as the internal control. The results are representative of two independent experiments, each with three corneas. *$P < 0.05$, **$P < 0.01$ (1-way ANOVA).

CECs (447.04- vs. 11.86-fold); its upregulation in the infected corneas was further augmented by *Isg15* deficiency (652.68-fold). At 3 dpi, while *Infa* remained unchanged, *Ifng* expression increased 6.24-fold in the infected corneas; this upregulation was totally abolished in *Isg15*$^{-/-}$ mouse corneas. The increase of *Il1b* was further elevated to 1868.43-fold in WT and augmented to 2590.37-fold in *Isg15*$^{-/-}$ mouse corneas. At 1 dpi and 3 dpi, the infection-induced expression of *Cxcl10* was elevated to 14.28- and 13.88-fold, respectively, and *Isg15* deficiency significantly suppressed its expression at both time points, 7.93 and 11.76, respectively. As for *Il36a*, the overall expression increased from 2.30 times at 6 hpi to 11.06 times at 1 dpi and 20.12 times at 3 dpi, although no differences were detected between WT and *Isg15*$^{-/-}$ mice in two late time points.

Infection-Induced ISG15 Expression Is Type 1 IFN Dependent in B6 Mouse Corneas

To assess whether *P. aeruginosa*–induced ISG15 expression is type 1 IFN dependent in the cornea,[32,33] we used *Ifnar* (type 1 IFN receptor) knockout mice. WT and *Ifnar*$^{-/-}$ mice were inoculated with 1.0×10^4 CFU *P. aeruginosa*, and the expression of *Isg15*, in comparison with *Il1b* and *Cxcl10*, was assessed at 1 dpi using qPCR (Fig. 4). In uninfected corneas, the levels of *Isg15* mRNA were significantly lower in *Ifnar*$^{-/-}$ than that in WT mouse corneas, while no differences were detected for *Il1b* and *Cxcl10*. Infection greatly upregulated all three genes in WT mice, and this upregulation was further augmented for *Il1b*, downregulated for *Cxcl10,* and unaffected for *Isg15 in Ifnar*$^{-/-}$ corneas. The levels of *Isg15* in *Ifnar*$^{-/-}$ corneas were significantly lower than the basal expression in WT, uninfected mouse corneas.

Ifnar Knockout Increases Severity of *P. aeruginosa* Keratitis and Abrogates ISG15-Induced Protection Against *P. aeruginosa* Infection

Having shown that *Isg15* expression in response to *P. aeruginosa* infection in B6 mouse cornea is type 1 IFN dependent, we next investigated the effects of *Ifnar* deficiency on the severity of the disease with or without exogenous ISG15 (Fig. 5). *Ifnar*$^{-/-}$ mice developed severe keratitis with average clinical scores of 6 and 11, compared to WT B6 mice of 3 and 7, at 1 and 3 dpi, respectively (Fig. 5A). While recombinant ISG15 protected the corneas of WT mice from *P. aeruginosa* infection, it exhibited partially protective effects on *Ifnar*$^{-/-}$ mice compared to the controls pretreated with BSA, with clinical scores of 2.2 and 5.4 at 1 and 3 dpi, respectively. Consistent with severity of keratitis, *Ifnar*$^{-/-}$ mice had a significantly higher bacteria burden (4.1×10^6) compared to WT corneas (1.2×10^6) at 3 dpi. While no viable bacteria were in ISG15-treated corneas, ISG15 pretreatment significantly reduced bacterial burden in *Ifnar*$^{-/-}$ mice (from 4.1×10^6 to 6.2×10^5) (Fig. 5B). *Ifnar* knockout resulted in an increase in MPO activity and IL-1β expression, whereas exogenous ISG15 decreased MPO activity and IL-1β expression (Fig. 5C, 5D). Interestingly, IFNγ was upregulated by *P. aeruginosa* infection and suppressed by *Ifnar* deficiency at 3 dpi with or without exogenous ISG15 (Fig. 5E).

ISG15 Alters the Expression of Chemokines and Defense Genes In Vitro and Elevates the Bactericidal Activity In Vivo

To understand the effects of secreted ISG15 on epithelial cells, we applied recombinant ISG15 to cultured primary HCECs and used qPCR to screen for cytokines with altered expression (Fig. 6A). While the expression of *Ifna* was not affected, their downstream genes *Ifng* and *Cxcl10* were slightly but significantly upregulated at different time points. ISG15 had no effects on *Il1b* expression (no shown) but surprisingly upregulated the expression of *Tslp* (thymic stromal lymphopoietin) at 2 hours poststimulation.

We next investigated whether exogenous ISG15 stimulates innate immunity by assessing in vitro inhibition of bacterial growth of ISG15-treated corneas (Fig 6B). The corneas of B6 mice treated with ISG15 or BSA were excised and homogenized. Naive corneas were also homogenized and mixed with recombinant ISG15 or BSA to serve as the controls. These homogenates were then incubated with 100 CFU of *P. aeruginosa* for 30 and 60 minutes. The homogenates of naive corneas with or without added ISG15 had similar numbers of bacteria at 30 (140 ± 20 vs. 143 ± 31) and 60 minutes (202 ± 40 vs. 200 ± 12). The corneas pretreated with BSA had similar numbers of bacteria (137 ± 12 at 30 minutes and 180 ± 45 at 60 minutes) to the naive corneas, whereas ISG15-treated corneal homogenates exhibited bacteriostatic activity with significantly less numbers of bacteria (94 ± 8 at 30 minutes and 109 ± 35 at 60 minutes).

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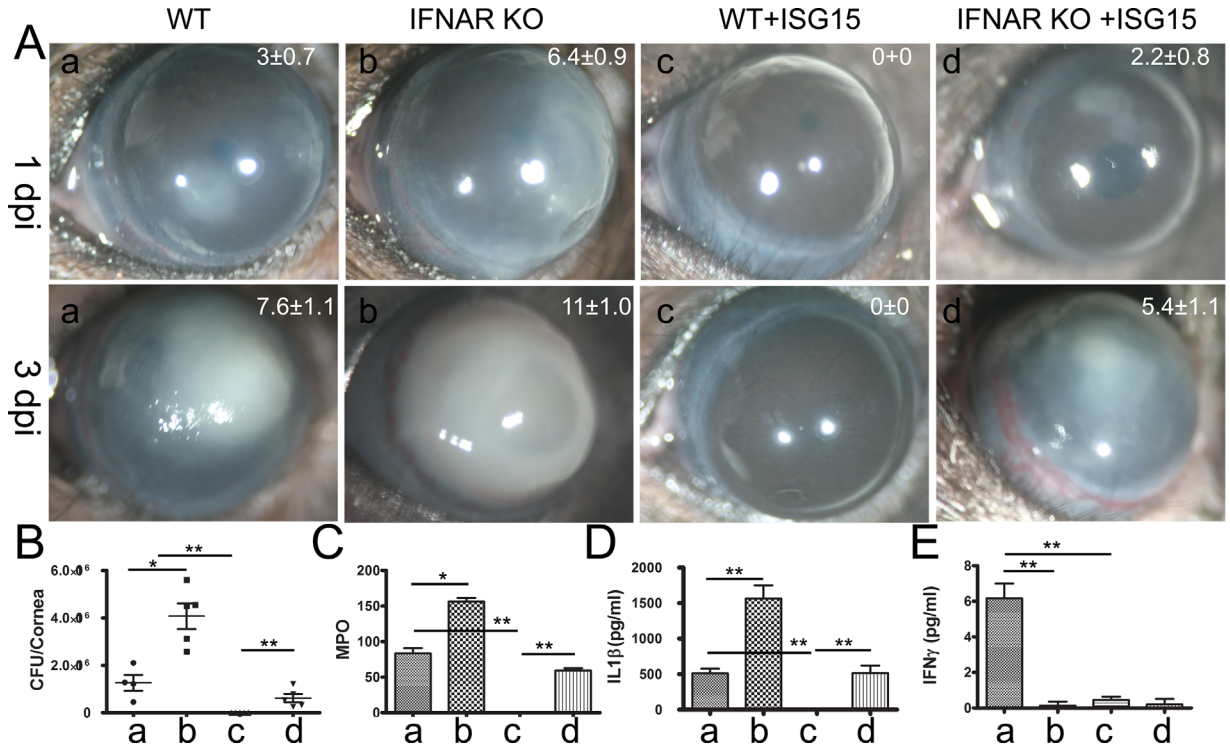

Figure 5. *Ifnar* deficiency increased the severity of *P. aeruginosa* keratitis in B6 mice. WT or *Ifnar*$^{-/-}$ mouse corneas were subconjuctivally injected with ISG15 or BSA at –6 hours, scarified, and inoculated with 1.0 × 10^4 CFU of *P. aeruginosa* at 0 hours. The infected corneas were photographed (**A**) at 1 and 3 dpi. At 3 dpi, the corneas were excised and subjected to CFU counting (CFU/corneas) (**B**) and MPO determination (units/cornea) (**C**) ($n = 5$). Another set of corneas was processed for ELISA determination of the levels of IL-1β (**D**) and IFN-γ (**E**) ($n \geq 3$). The results are representative of two independent experiments. *$P < 0.05$, **$P < 0.01$.

ISG15 Modulates Innate Immune Response Through LFA-1

Recently, the ICAM1 receptor LFA-1 was reported to be the receptor for extracellular ISG15 to stimulate IFNγ secretion in NK cells.[34] We next investigated whether IFA1 is involved in ISG15-induced protection from *P. aeruginosa* keratitis. A286982 is a potent inhibitor of the LFA-1 (integrin $\alpha_L\beta_2$)/ICAM1 interaction (IC50 = 44 nM). B6 mouse corneas were subconjunctivally injected with ISG15 and ISG15 + A286982 and then inoculated with 1.0 × 10^4 *P. aeruginosa*. As shown in Figure 7, A286982 alone significantly increased severity of *P. aeruginosa*, and the presence of ISG15 did not significantly improve the outcome of keratitis assessed by clinical scores, CFU, and MPO activities, suggesting extracellular ISG15 functions through LFA-1.

Discussion

In this study, we investigated the role of ISG15 in protecting the corneas from *P. aeruginosa* infection. We showed that while ISG15 conjugates are present, its free forms, cellular and extracellular, are increased after H-K *P. aeruginosa* challenge of cultured HCECs. We demonstrated that *Isg15* expression in *P. aeruginosa*–infected corneas is type 1 IFN dependent. *Isg15* deficiency not only greatly increased corneas' susceptibility to *P. aeruginosa* infection but also exuberated infection-induced IL-1β expression while abolishing IFNγ and reducing CXCL10 expression. Exogenous ISG15, on the other hand, prevents the corneas from and attenuates progression of *P. aeruginosa* keratitis in *Ifnar*$^{-/-}$ mice. The ability of ISG15 to enhance an innate immune defense against *P. aeruginosa* is related to its ability to promote antimicrobial peptide expression and bactericidal/bacteriostatic activity in the cornea. Finally, extracellular ISG15 functions through its newly identified receptor LFA-1. Taken together, our study identifies an additional physiologic function of ISG15 as an immunoregulator to participate in innate immune regulation through LFA-1 and suggests ISG15 might be used as an adjunctive therapy to treat bacterial keratitis.

Type I IFNs, IFN-α and IFN-β, play a pivotal role in regulating pathogen invasion through the induction of hundreds of ISGs.[19] Among these ISGs, ISG15 is one of the most strongly and abundantly induced ISGs in response to microbial infection.[35,36] Our in vitro study revealed that H-K *P. aeruginosa* upregulates ISG15 expression and secretion in HCECs. The pattern of ISG15 conjugates remains largely unchanged during H-K *P. aeruginosa* challenge, suggesting that ISGylation may not be involved in innate protection against bacterial infection. We recently showed that ISG15 acts as an immunomodulator in the cornea and plays a critical role in controlling fungal keratitis.[16] As for bacterium infection, ISG15 has been shown to play a protective role in defending the infection of *Salmonella typhimurium*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*,[37–39] all of which are invasive bacteria. In a mouse model of *Toxoplasma gondii* infection, increased ISG15 expression and secretion are dependent on active parasites' invasion and replication in cells.[18] *P. aeruginosa* (strain ATCC 19660) is noninvasive and cytotoxic; our study shows for the first time, to our knowledge, a protective immunomodulatory role of

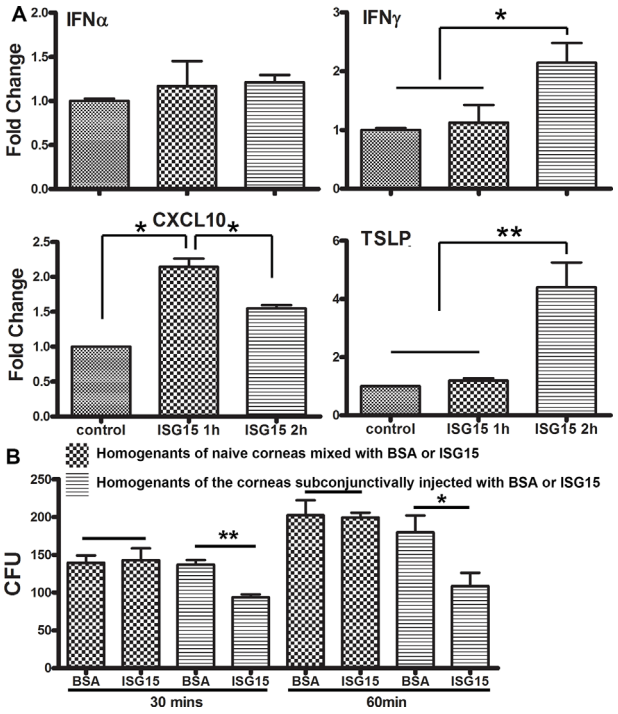

Figure 6. Recombinant ISG15 affects gene expression in PHCECs (Primary Human Corneal Epithelial Cells) and in vitro bactericidal activity of ISG15-treated corneas. Cultured primary HCECs were treated with human recombinant ISG15 (200 ng) and collected at 1 or 2 hours and subjected to RNA isolation and real-time PCR analysis (**A**). Naive B6 mouse corneas with subconjuctival injection or ISG15, with BSA as the control, for 6 hours, excised, minced, and homogenized in 100 µL PBS with a TissueLyser (Retch). A total of 200 ng recombinant ISG15 or BSA was added to the naive corneal homogenates. *P. aeruginosa* (100 CFU) in 400 µL PBS was incubated with corneal homogenates for 30 and 60 minutes at 37°C. At the end of incubation, the samples were subjected to bacterial plate-colony counting. The results are representative of two independent experiments, each with three samples (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$ (1-way ANOVA).

ISG15 for noninvasive bacteria in a mucosal tissue. Hence, upregulation and/or secretion of ISG15 might be a universal protective response to tissue infection regardless of the types of pathogens and tissues.

The protective effects of ISG15 were further illustrated by using *Isg15* KO mice. *Isg15* deficiency greatly increased the severity *P. aeruginosa* keratitis detectable at 1 dpi and more apparent at 3 dpi. There was no detectable expression of IFNγ while the expression of type 1 IFNs was not affected in the corneas of *Isg15*$^{-/-}$ mice. The expression of proinflammatory cytokine IL-1β was augmented in infected *Isg15*$^{-/-}$ corneas, consistent with elevated severity of keratitis. The infection-induced expression of *Cxcl10*, on the other hand, was suppressed fully at 6 hpi but partially at 1 and 3 dpi, suggesting that unlike IFNγ, *Cxcl10* expression partially depends on ISG15. This was further verified in Ifnar$^{-/-}$ mice in which the infection-induced *Cxcl10* expression was partially suppressed while *Isg15* expression was totally inhibited at 1 dpi. It is plausible that type 1 IFN-independent *Cxcl10* may be partly insensitive to *Isg15* deficiency in B6 mouse corneas. Hence, while ISG15, CXCL10, and IFNγ were all downstream of type 1 IFNs, ISG15 functioned as an upstream mediator for IFNγ and CXCL10. Consistent with the disturbed cytokine expression, *Ifnar* deficiency significantly increased the susceptibility of B6 mouse corneas to *P. aeruginosa* infection. Administration of ISG15 in WT mice resulted in total protection of the corneas from *P. aeruginosa* infection and greatly reduced severity of *P. aeruginosa* keratitis in *Ifnar*$^{-/-}$ mice, suggesting that extracellular ISG15 contributes significantly to IFN/IFNAR (interferon-α/β receptor)-dependent innate immunity and host defense against *P. aeruginosa* infection. Moreover, although the presence of ISG15 in *Ifnar*$^{-/-}$ mouse corneas exhibited profound effects on decreasing CFU, MPO activity, and IL-1β expression, it was unable to stimulate IFNγ expression in these mice, indicating that extracellular ISG15 is necessary but not sufficient for the induction of IFNγ expression in *P. aeruginosa*–infected mouse corneas.

How might ISG15 mediate the innate killing of invading pathogens, resulting in the eradication of inoculated *P. aeruginosa* in B6 mouse corneas? The evidence has shown that ISG15 conjugation inhibits many viruses, including influenza A and B viruses, Sindbis virus, human immunodeficiency virus (HIV) 1, herpes simplex 1, and murine herpesvirus.[20,40–45] Free ISG15 also has an antiviral function against chikungunya, HIV, and Ebola virus.[36,45–47] In contrast to antiviral activity, our data showed that free ISG15 had no effects on *P. aeruginosa* growth in vitro. However, ISG15 stimulated the expression of human *Tslp* and *Cxcl10* at mRNA levels in cultured HCECs but not *Il1b*. TSLP is a pleiotropic cytokine and also displays potent antimicrobial activity, exceeding that of many other known antimicrobial peptides.[48] As for CXCL10, in addition to being a chemokine to recruit NK cells in infected cornea,[49] it also acts as an antimicrobial molecule, killing pathogens such as multidrug-resistant gram-negative pathogens[50] and *Candida albicans*.[48,49,51–53] Hence, we propose that ISG15 activation–stimulated CXCL10 and/or TSLP are the effectors that play a role in innate defense during the early period of *P. aeruginosa* infection of the cornea. This is further supported by in vivo stimulation of B6 mouse corneas with ISG15, resulting in a lower number of CFUs than that treated with BSA at 30 and 60 minutes of in vitro culture, indicating elevated bacteriostatic activity in the treated corneas.

Although extracellular ISG15 has been shown to be an immunomodulator to elicit IFNγ secretion from lymphocytes since 1991,[54] the basis of ISG15 signaling and the identification of a putative cell surface receptor were not resolved until 2017. Swaim et al.[34] reported that ISG15 stimulated IFNγ secretion from different cells in an LFA-1 (CD11a/CD18; αLβ2 integrin)–dependent manner. To date, no follow-up studies were reported to support ISG15 as a ligand of LFA-1. Our study, using an LFA-1 inhibitor and exogenously administrated ISG15, revealed that blocking LFA-1 signaling greatly reduced ISG15-mediated protection in *P. aeruginosa* keratitis, indicating that ISG15 functions in the corneas through LFA-1 signaling. Since ICAM1 also uses LFA-1 as the receptor in innate immune cells, how ISG15 and ICAM1 act on LFA-1 in controlling infection and inflammation warrants further investigation.

We conclude that ISG15 plays a protective role in corneal innate immunity and protects against *P. aeruginosa* infection. ISG15 may act in an LFA-1 signaling-dependent, CXCL10 and TSLP expression-related manner. ISG15 may be used as an adjective therapy to enhance innate mucosal protective immunity against a broad range of pathogens.

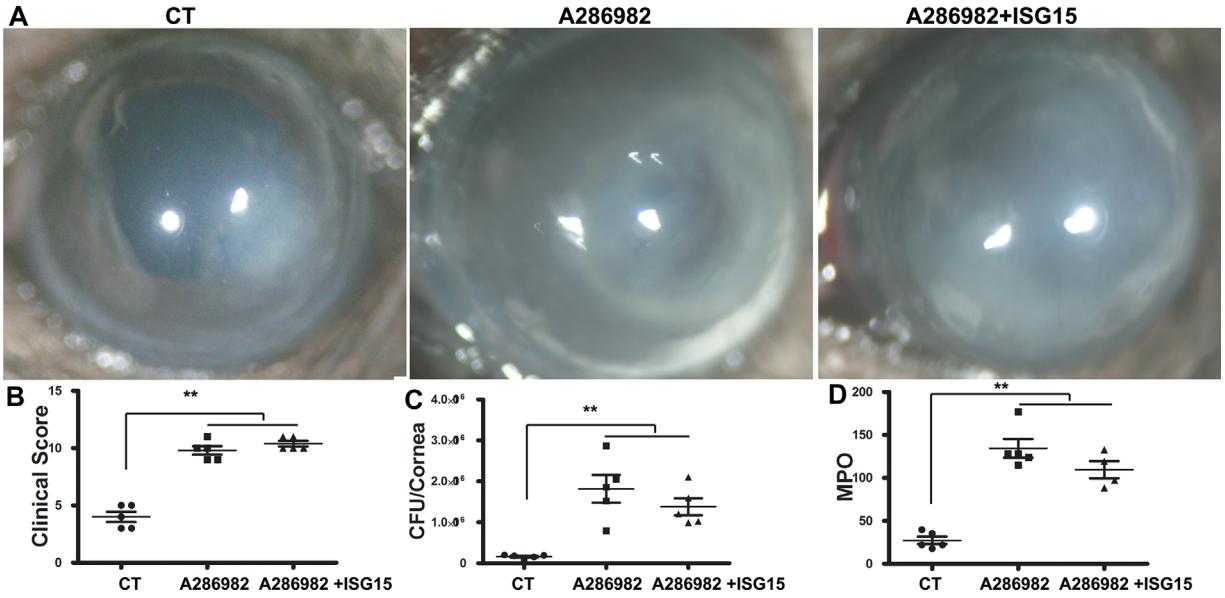

Figure 7. ISG15-induced protection against *P. aeruginosa* infection in B6 mouse corneas is LFA-1 dependent. B6 mouse corneas were subconjuctivally injected IFA1 inhibitor A286982 or A286982 plus ISG15 at –6 h, scarified, and inoculated with 1.0×10^4 CFU of *P. aeruginosa* at 0 hours. The infected corneas were photographed (**A**) and clinically scored (**B**) at 1 dpi. The corneas were excised and subjected to CFU counting (CFU/corneas) (**C**) and MPO determination (units/cornea) (**D**) ($n = 5$). The results are representative of two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ (1-way ANOVA).

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