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Toll-like receptor 2 (TLR2) engages endoplasmic reticulum stress sensor IRE1α to regulate retinal innate responses in *S. aureus* endophthalmitis

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Running title: IRE1α regulates innate response in bacterial endophthalmitis

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ABSTRACT

Endoplasmic reticulum (ER) stress response has been implicated in a variety of pathophysiological conditions, including infectious and inflammatory diseases. However, its contribution in ocular bacterial infections, such as endophthalmitis, which often cause blindness is not known. Here, using a mouse model of *Staphylococcus* (*S.*) *aureus* endophthalmitis, our study demonstrates the induction of inositol-requiring enzyme 1α (IRE1α) and splicing of X-box binding protein-1 (*Xbp1*) branch of the ER-stress pathway, but not the other classical ER stress sensors. Interestingly, *S. aureus* induced ER stress response was found to be dependent on Toll-like receptor 2 (TLR2), as evident by reduced expression of IRE1α and *Xbp1* mRNA splicing in TLR2 knockout mouse retina. Pharmacological inhibition of IRE1α using 4μ8C or experiments utilizing IRE1α$^{-/-}$ macrophages revealed that IRE1α positively regulates *S. aureus* induced inflammatory responses. Moreover, IRE1α inhibition attenuated *S. aureus*-triggered NF-κB, p38, and ERK pathways activation and cells treated with these pathway-specific inhibitors reduced *Xbp1* splicing, suggesting a positive feedback inhibition. *In vivo*, inhibition of IRE1α diminished intraocular inflammation and reduced PMN infiltration in mouse eyes, but, increased bacterial burden and caused more retinal tissue damage. These results revealed a critical

role of the IRE1α/XBP1 pathway as a regulator of TLR2 mediated protective innate immune responses in *S. aureus* induced endophthalmitis.

KEY WORDS: *S. aureus*, Endophthalmitis, Retina, Microglia, Reactive oxygen species (ROS), Inflammation, Endoplasmic reticulum (ER) stress, Toll-like receptor 2 (TLR2).

ABBREVIATIONS

TLR2, Toll-like receptor 2; ER, Endoplasmic reticulum; IRE1α, Inositol-requiring enzyme 1α; XBP1, X-box binding protein-1; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, Reactive oxygen species; UPR, Unfolded protein response; PERK, protein kinase RNA-like ER kinase; ATF6, activating transcription factor 6; RNA, Ribonucleic acid; WT, Wild type; DLAR, Division of laboratory animal resources; ARVO, Association for Research in Vision and Ophthalmology; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; MOI, Multiplicity of infection; BMDM, Bone marrowderived macrophages; mM, millimolar; EDTA, Ethylenediaminetetraacetic acid; M-CSF, Macrophage colony-stimulating factor; PCR, Polymerase Chain reaction; ELISA, Enzymelinked immunosorbent assay; TNFα, tumor necrosis factor-alpha; IL-1β, Interleukin-1beta; IL6, Interleukin-6; MIP2, Macrophage Inflammatory Protein 2; CXCL2, Chemokine (C-X-C motif) ligand 2; KC, Keratinocyte chemoattractant; CXCL1, chemokine (C-X-C motif) ligand 1; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; H&E, hematoxylin and eosin; PMN, Polymorphonuclear cells; BSA, Bovine serum albumin; HRP, Horseradish peroxidase; LPS, Lipopolysaccharides; SA, *S. aureus*; LTA, Lipoteichoicacid; PGN,Peptidoglycan.

INTRODUCTION

Staphylococcus (S) aureus has long been recognized as an important bacterial pathogen causing various human diseases including infection of the bloodstream, skin, bone and joints as well as pneumonia (1-3). Aside from the aforementioned manifestations, *S. aureus* remains the leading cause of ocular diseases, such as bacterial endophthalmitis, which often results in poor prognosis even after treatment (4). Mostly, ocular trauma or surgical procedures predispose the eye to develop bacterial endophthalmitis (5, 6). Visual prognosis mainly depends on the virulence properties of the causative organism, visual ingenuity, and the efficacy of the antimicrobial treatment regime (7). Eye being an immunoprivileged organ, the retina is highly susceptible to host-induced inflammatory damage along with the injury caused by pathogens virulence factors. Previously, we have shown that retinal cells including Müller glia, microglia, and photoreceptors cells mount a massive innate immune response against *S. aureus* infection that could contribute to retinal damage in addition to providing protection against pathogen (8-10). Studies from both our laboratory and others have sought to elucidate the role of Toll-like receptors (TLRs) in the initiation of innate defense mechanisms in bacterial endophthalmitis (4, 8, 11-15). Of particular interest is TLR2, which has been found to orchestrate protective retinal innate immune responses in *Staphylococcal* endophthalmitis via modulating intraocular inflammation, and induction of antimicrobial peptides (4, 8-11, 15).

Endoplasmic reticulum (ER) is an intricate cellular organelle present in eukaryotic cells. The ER is a major site for protein synthesis and maturation and is involved in the processing of secretory and membrane proteins (16, 17). A wide variety of cellular conditions, including glucose deprivation, disruption of calcium homeostasis, and both viral and bacterial

infections, have been implicated as the causative agent behind the influx of unfolded or misfolded peptides which results in an ER stress response. To deal with this stress, the ER has evolved a set of signal transduction pathways that are collectively termed as unfolded protein response (UPR). For sensing ER stress, three major transmembrane transducers have been identified: Protein kinase RNA-like ER kinase (PERK), Inositol requiring enzyme (IRE1α), and activating transcription factor 6 (ATF6). Compared to other stress sensors, IRE1α is the most conserved signaling branch. IRE1α activation induces unconventional splicing of a 26-nucleotide intron from the RNA encoding X-box binding protein 1 (XBP1) to convert into mature XBP1s which then acts as a transcription factor (18). The IRE1α-XBP1 branch is evolutionary conserved from yeast to human and is essential for mammalian developmental processes (19). The cytoplasmic portion of IRE1α has both kinase and endonuclease activities, whereas the luminal domain can detect unfolded proteins. The IRE1α induced XBP1s then activates downstream genes, including the ER chaperones ERDj4, GRP78 (BiP), and PDI which are known to play a central role in restoring cellular ER homeostasis and promoting cell survival (20, 21). If ER stress is sustained for too long, it can result in persistent inflammation and eventual cell death (22).

Transcriptional induction of the *Xbp1* mRNA precursor after TLR stimulations in human macrophage, as well as mouse lung tissues during *Mycobacterium* and *Klebsiella* infection, have been reported (23, 24). ER stress response has been shown to either support or hamper disease progression by regulating inflammatory host defense pathways depending on the cell types, disease model, and the ER stressor (25). It has been shown that some intracellular pathogens utilize ER stress signaling as a protective mechanism for their intracellular growth and UPR induction is beneficial to the infection (26). Previously, TLR2

and TLR4 ligands mediated activation of ER stress response have been shown, which demonstrates a novel mechanism of IRE1 activation independent of protein misfolding in the ER lumen (27, 28). Nevertheless, the role of ER stress response in ocular infections, especially in bacterial endophthalmitis remains largely unexplored. Recently, we performed a transcriptomic analysis in *S. aureus* infected mouse retina (29) and discovered dysregulation of genes modulating ER stress response including *Xbp1* and *Bip*. Therefore, we intitated this study to investigate the role of ER stress in the bacterial ocular infection.

In the current study, we show that *S. aureus* infection in the eye specifically induces the IRE1α/XBP1 axis of ER stress. Notably, we found that IRE1α is essential for the induction of TLR2-mediated innate inflammatory responses and that the abrogation of this pathway is detrimental. In accordance, IRE1α inhibition resulted in increased bacterial burden and retinal tissue damage in the eye. These results suggest that IRE1α/XBP1 signaling is a protective host response in ocular infections.

MATERIALS AND METHODS

Animals

C57BL/6 [wild type (WT)] mice (both male and female, 6-8 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME). TLR2$^{-/-}$ breeders were purchased from Jackson Laboratory, bred in-house, and maintained in a pathogen-free, restricted-access Division of Laboratory Animal Resources (DLAR) facility at Kresge Eye Institute. *IRE1α$^{flox/flox}$* and myeloid cell-specific IRE1$\alpha^{-/-}$ mice (28) were provided by Dr. Kezhong Zhang (Center for Molecular Medicine and Genetics, Wayne State University). All animals were maintained

on a 12 h light 12 h dark cycle at 22℃ temperature and provided free access to the tap water and LabDiet rodent chow (PicoLab; LabDiet, St. Louis, MO). All the procedures were conducted in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University.

Cell culture

An immortalized mouse microglia (BV2) cell line was maintained in low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS and a penicillin-streptomycin cocktail (Invitrogen, Carlsbad, CA) in a humidified 5% CO_2 incubator at 37℃. Before treatment, cells were cultured in antibiotic-free and serum-free DMEM for 18 h (growth factor starvation). Cells were either stimulated with TLR agonist Pam3CysSK4 (10 µg/ml) or infected with *S. aureus* RN6390 multiplicity of infection (MOI) 10:1 for 8h.

Induction of *S. aureus* endophthalmitis

Endophthalmitis was induced in mice as described previously (4, 10). Briefly, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (ketamine, 100-125 mg/kg: xylazine, 10-12.5 mg/kg). Under an ophthalmoscope, mice left eyes were injected intravitreally with *S. aureus* strain RN6390 (5000CFU/eye) using a 34G needle attached to a 10µl syringe (WPI). Contralateral eyes injected with sterile PBS served as control. At the desired time points post-infection; enucleated eyes or neural retina were subjected to bacterial growth determination, inflammatory cytokines/ chemokines assays, Western blotting, and histology as described in the following sections.

Isolation of bone marrow-derived macrophage (BMDM)

Bone marrow-derived macrophages (BMDM) were isolated as described previously (30, 31). Briefly, bone marrow cells from *IRE1α*$^{flox/flox}$ and myeloid cell-specific IRE1α KO mice were flushed from femurs and tibias using RPMI media containing 10% FBS and 0.2 mM EDTA. RBCs were lysed by adding a hypotonic solution of 0.2% NaCl for 20 s, followed by the addition of 1.6% NaCl. Bone marrow cells were pelleted by centrifugation at 400 x g for 5 min followed by a wash with RPMI media. Cells were resuspended, counted, and cultured in RPMI media supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 ng/ml M-CSF at 37℃ in 5% CO2 for macrophage differentiation. Six days post-differentiation ~1×10^6 BMDM/ml were seeded in 6 well tissue-culture plates for *in-vitro* experiments.

RNA extraction and Polymerase chain reaction (PCR)

Total RNA was extracted from both mouse neural retina and BV2 cells using TRIzol per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reversed transcribed using Maxima first-strand cDNA synthesis kit per manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). cDNA was amplified for a gene of interest by PCR using mouse-specific primers. PCR products along with housekeeping internal control GAPDH, were subjected to electrophoresis on 2.5% or 1.2% agarose gel according to the size of the PCR amplicons. For XBP1 splicing detection, both, unspliced and spliced forms of XBP1 mRNA species were resolved using high-percentage (2.5%) agarose gel electrophoresis which is often used to detect IRE1α endonuclease activity.

Images of ethidium bromide-stained gels were captured using a digital camera (EDAS 290 system, Eastman Kodak, Rochester, NY).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to quantify the levels of cytokines/chemokines in mice ocular tissue as well as the cells conditioned media. Briefly, mouse eyes were enucleated and homogenized in sterile PBS by stainless steel beads using Tissue lyser (Qiagen, Valencia, CA) followed by centrifugation at 15,000 x g for 15 min. Total protein was estimated using the Micro BCA™ protein estimation kit (Thermo Scientific, Rockford, IL, USA) per manufacturer's instructions. ELISA was performed for tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) (BD biosciences, San Diego, CA, USA) and macrophage inflammatory protein 2 (MIP2/CXCL2) and CXCL1/KC (R&D systems, Minneapolis, MN, USA) per manufacturer's instructions.

Histology and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Mice eyes were enucleated at desired time points for histopathological examination and fixed in 10% formalin. Embedding, sectioning and hematoxylin and eosin (H&E) staining were performed by Excalibur Pathology Inc. (Oklahoma City, OK, USA). All retinal H&E sections were observed with a light microscope (400 X magnification). For TUNEL staining, the eyes were fixed in Tissue-Tek OCT (Sakura, Torrance, CA, USA) and 6-8 µm thick sagittal sections were collected from each eye and mounted onto microscope slides. Retinal

sections were used for the TUNEL staining using ApopTag® Fluorescein In situ Apoptosis Detection Kit according to the manufacturer's instruction (Millipore, Billerica, MA, USA).

Polymorphonuclear cells (PMNs) infiltration

To determine the PMNs infiltration in mice retina, flow cytometry was used as described previously (32). Briefly, following euthanasia, retinas were isolated and digested with Accumax (Millipore, MA, USA) for 10 minutes at 37℃. A single-cell suspension was prepared by triturating the retina using a 23-G needle/syringe and filtered through a 40µm cell strainer (BD Falcon, San Jose, CA, USA). To reduce the non-specific binding of antibodies, cells were incubated with Fc Block (BD Biosciences) for 30 minutes. After washing with 0.5% BSA, cell suspensions were incubated with conjugated monoclonal antibodies CD45-PECy5, Ly6G-FITC, and respective isotype controls (BD Biosciences) in dark for 30 minutes. After washing, cells were acquired and analyzed using the Accuri C6 flow cytometer and software (BD Biosciences, San Jose, CA, USA) respectively.

Immunoblotting

For immunoblotting, cells were lysed using radioimmunoprecipitation (RIPA) lysis buffer containing a protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Mice neural retinas were lysed by sonication in PBS containing a protease and phosphatase inhibitor cocktail. Total protein concentration was determined, and 30-40 µg protein was used for blotting. Denatured proteins were resolved in a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (0.44µm) (Bio-Rad Laboratories). Following blocking, the blots were incubated with anti-phospho (Ser-51)-

IRE1α, anti-total IRE1α (1:1000) (Cell signaling Technology, Boston, MA) and anti-β-actin (1:5000) (Sigma-Aldrich, St. Louis, MO) antibodies overnight at 4ºC. Following washing, blots were incubated with goat anti-rabbit/ mice-IgG-HRP conjugate (BioRad, Hercules, CA). Protein bands were developed using SuperSignal West Femto Chemiluminescent Substrate and visualized using iBright FL1500 Imaging Systems (ThermoFisher Scientific, Rockford, IL). β-actin was used as a control for protein loading. Quantification of the intensity of bands was performed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/, 1997-2009).

Immunostaining

Immunostaining was performed as described earlier (33, 34). Briefly, cells were cultured on four well glass chamber slides (Fisher Scientific, Rochester, NY) and pre-treated with 4μ8C (IRE1α inhibitor) 1h before *S. aureus* challenge. Following stimulation, cells were washed three time with PBS and fixed in 4% paraformaldehyde for 15 min. Cells were permeabilized with an ethanol: acetic acid mixture (2:1) at -20℃ for 10 min. and washed. The fixed cells were blocked in 1% (w/v) BSA for 1h at room temperature followed by incubation with primary antibodies (1:100 dilution) overnight at 4℃. Cells were washed with PBS and incubated with specific fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:200 dilutions) for 1h at room temperature. Following incubation cells were washed with PBS and mounted in Vectashield anti-fade mounting medium with DAPI (Vector Laboratories). Slides were visualized using an Eclipse 90i fluorescence microscope (Nikon, Melville, NY)

Statistical analysis

Statistical analysis was performed using GraphPad Prism V8 (GraphPad Software, La Jolla, CA). All data has been expressed as means ± SD unless indicated otherwise. Unpaired student t-test or One-way ANOVA was used for comparisons followed by Dunnett's' posthoc test wherever applicable. A *P*-value of <0.05 was considered statistically significant. All experiments were performed at least three time unless indicated otherwise.

RESULTS

ER stress is induced during *S. aureus* endophthalmitis

Bacterial infections have been shown to trigger an unfolded protein response (UPR) (35, 36) resulting in the activation of the ER-transmembrane protein IRE1α. Although multiple targets for the IRE1α endonuclease have been identified, the splicing of *Xbp1* mRNA is the main under infectious and inflammatory conditions (37). To assess whether IRE1α-XBP1 or other ER stress sensors are induced in bacterial endophthalmitis, we analyzed transcriptomic data that had been previously published (29). Indeed, our data showed a time-dependent induction of *XBP1* (**Fig 1A**, upper panel) and *BiP* (**Fig 1A**, lower panel) mRNA transcripts upon *S. aureus* infection by qRT-PCR. To further confirm our microarray data, we performed independent experiments and found *S. aureus* infection induced the expression of IRE1α and the splicing of *XBP1* mRNA in mice retina, indicating the onset of ER stress response (**Fig 1B**). However, the expression of CHOP, WSF, ER-localized DnaJ homologue 4 (ERDj4), and the disulfide isomerase PDI did not change noticeably in infected retinal tissue as compared to control (**Fig 1B**), indicating that ATF6 and PERK pathways are

196 unlikely involved in *S. aureus* induced ER stress response in the retina (38, 39). These
197 observations led us to focus on the IRE1α mediated ER stress pathway in *S. aureus*
198 endophthalmitis.

199 To evaluate the specificity of the bacterial-induced IRE1α-XBP1 axis of ER stress we used
200 a potent pharmacological inhibitor of IRE1α, 4μ8C, which inhibits substrate access to the
201 active site of IRE1 leading to the inactivation of *Xbp1* splicing and IRE1-mediated mRNA
202 degradation (40). To investigate this, first, we performed a dose-response study using 4μ8C
203 on mouse BV2 microglia cells (10) challenged with *S. aureus* (**Fig S1A**). Because all the
204 dosages of 4μ8C tested showed a reduction of *XBP1* mRNA splicing (XBP1s), we decided
205 to use 100 nM of this inhibitor for the remainder of the study.

206 ***S. aureus* induces IRE1α mediated ER stress response via TLR2**

207 In the eye, *S. aureus* has been shown to invoke retinal innate responses through TLR2
208 signaling (4, 10, 11) and TLRs have been implicated in triggering ER stress (26, 27), we
209 sought to decipher the link between TLR2 and IRE1α activation in our disease model, which
210 is currently unknown. To establish the role of TLR2 signaling in regulating IRE1α mediated
211 ER stress response, *S. aureus* endophthalmitis was induced in wild type (WT) C57BL/6,
212 TLR2$^{-/-}$, and MyD88$^{-/-}$ mice with or without 4μ8C pretreatment. Our data shows *S. aureus*
213 infection-induced activation of IRE1α and prominent splicing of *Xbp1* transcripts in WT and
214 MyD88$^{-/-}$ mice and the response was attenuated by 4μ8C treatment. In contrast, infected
215 TLR2$^{-/-}$ mouse retina did not show the splicing of XBP1 (**Fig 2A**). We further confirmed the
216 IRE1α activation at protein levels in retinal tissue of *S. aureus* infected WT, TLR2$^{-/-}$ and
217 MyD88$^{-/-}$ mice by western blotting. Our results show that *S. aureus* significantly induced the

phosphorylation of IRE1α in WT and MyD88$^{-/-}$ mice whereas, its levels were significantly lower in TLR2$^{-/-}$ mice (**Fig 2B**). These results indicate that TLR2 mediates IRE1α-mediated-ER stress response in *S. aureus* endophthalmitis.

For *in vitro* studies, we used mouse BV2 microglia which have been shown to respond to *S. aureus* challenge analogous to that of primary retinal microglia (10). To examine the role of TLRs in IRE1α activation in our model, BV2 microglia were challenged with the TLR2 agonist, Pam3CSK4, and the TLR4 agonist, LPS, in the presence and absence of IRE1α inhibitor 4μ8C; live *S. aureus* (SA) was used as a positive control. Our results show all agents (Pam3CSK4, LPS, and SA) induced the expression of IRE1α as well as the splicing of *XBP1* and that this response was reduced by 4μ8C treatment (**Fig 2C**). We also examined the activation of classical ER-stress response and found no marked induction of CHOP, ERDj4, and PDI expression in response to the Pam3CSK or *S. aureus* challenge (**Fig 2C**). Induction of IRE1α and its inhibition by 4μ8C was confirmed at the protein level by western blotting (**Fig 2D**) and immunostaining (**Fig S1B**), which displayed reduced phosphorylation of IRE1α upon 4μ8C treatment in BV2 microglial cells.

In addition to specific TLR ligands, we assessed the effect of *S. aureus* virulence factors (α-toxin, LTA, PGN) on ER stress response and observed that they also modestly induced the expression of IRE1α and XBP1 splicing (**Fig S2A**).

IRE1α regulates *S. aureus* and TLR2 ligand-induced inflammatory response

TLR mediated ER stress response has been linked to the elicitation of innate responses in various pathological conditions (27, 41, 42). Hyperglycemia-induced ER stress has also

been implicated in retinal Müller glia-derived inflammatory response in diabetic retinopathy (43). To assess the functional role of IRE1α activation in *Staphylococcal* endophthalmitis, we evaluated inflammatory mediators in mice eyes and cultured BV2 microglial cells. As expected, *S. aureus* induced the production of key inflammatory cytokines, IL-1β, TNF-α, and the chemokine MIP2 in retinal tissue, whereas their levels were significantly reduced in mouse eyes pre-treated with the IRE1α inhibitor 4μ8C (**Fig 3A**). Similarly, pretreatment of BV2 microglia with 4μ8C significantly attenuated inflammatory mediators induced by *S. aureus* and Pam3CSK4 (**Fig 3B**).

Next, to further solidify the role of IRE1α in an *S. aureus* induced inflammatory response, we used bone marrow-derived macrophages (BMDM) from IRE1$\alpha^{flox/flox}$ and myeloid cell-specific IRE1$\alpha^{-/-}$ mice. *S. aureus* challenge of IRE1$\alpha^{flox/flox}$ BMDM resulted in robust production of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) but this was not the case in IRE1$\alpha^{-/-}$ BMDM (**Fig 3C**). Collectively, these results support the hypothesis that IRE1α mediates an innate inflammatory response in both *Staphylococcal* endophthalmitis and cultured immune cells (microglia and macrophages).

Inhibition of IRE1α attenuates *S. aureus* induced NF-κB and MAPK signaling

Because NF-κB and other MAP kinases, such as ERK and p38 are known to regulate the inflammatory response in *S. aureus* endophthalmitis (10, 30, 44), we decided to assess their link with IRE1α activation (45). *Staphylococcal* endophthalmitis was induced in WT mouse eyes pretreated with 4μ8C, and western blot was performed to detect IκB and MAPKs in retinal tissue lysates. Our results show that *S. aureus* induced phosphorylation of IκB, ERK1/2, and p38 proteins, whereas, IRE1α inhibition reduced the activation of these

signaling molecules (**Fig 4A**). Similarly, the pathway-specific inhibitors for IκB, ERK1/2, and p38 reduced *S. aureus*-induced *Xbp1* splicing (**Fig 4B**), indicating the existence of a feedback inhibition system among these pathways. The activity of these pathway inhibitors was validated by assessing *S. aureus* induced mRNA expression of the inflammatory cytokine *Il-1β* (**Fig S2B**). Together, these results suggest that IRE1α is an upstream regulator of *S. aureus*-induced NF-κB and MAPKs signaling.

S. aureus **induced ROS activates IRE1α-XBP-mediated ER stress response**

A correlation between ROS generation and the induction of ER stress has been shown in several pathological conditions (46-48). Previously, we have shown that *S. aureus* induces ROS generation in retinal cells (9). Therefore, we sought to investigate whether *S. aureus* induced ROS production plays any role in the IRE1α mediated ER stress response. To investigate this, we used Diphenyleneiodonium (DPI), an NAD(P)H oxidase inhibitor, and a potent ROS blocker. Our data showed DPI pretreatment in BV2 microglia, reduced *S. aureus*-triggered IRE1α expression as well as *Xbp1* splicing (**Fig 5A**). Moreover, the conditioned media from DPI treated cells showed reduced accumulation of inflammatory mediators (**Fig 5B**). This observation indicates that *S. aureus*-induced ROS generation contributes to the induction of ER stress, and blocking ROS production could prevent *S. aureus*-induced ER stress.

IRE1α inhibition results in increased bacterial burden and tissue damage in the eye

ER stress can either promote or impede disease progression depending on the disease model, cell types, and the ER stress sensors involved (49-51). Studies have shown that

some intracellular pathogens use ER stress signaling as a protective mechanism for their intracellular growth, making IRE1α induced UPR beneficial for the spread of infection (25, 52, 53). Since found that *S. aureus* induced IRE1α activation and *Xbp1* splicing both *in vivo* and *in vitro*, and IRE1α inhibition diminished pro-inflammatory cytokine production, we sought to determine the effect of IRE1α inhibition (4μ8C treatment) on disease progression. First, we assessed the intraocular bacterial burden, and unexpectedly, we found that IRE1α inhibition resulted in higher bacterial load in the eyes as compared to *S. aureus* alone injected eyes. (**Fig 6A**). To assess *S. aureus*-induced disease pathology, we performed histological analysis and our data showed increased retinal damage (retinal folding and disintegration of retinal layers), and fibrin formation in 4μ8C-treated eyes compared to eyes only infected with *S. aureus* (**Fig 6B**). This observation was confirmed by TUNEL staining of retinal sections which showed more TUNEL positive cells in eyes treated with the IRE1α inhibitor, 4μ8C (**Fig 5C**), indicating increased retinal cell death. We also performed flow cytometry to assess PMN infiltration and found significantly reduced PMN infiltration in the retina upon IRE1α inhibition (**Fig 6D**). Altogether, these results indicate that IRE1α-mediated ER stress is essential in controlling disease pathology in bacterial endophthalmitis.

DISCUSSION

This study demonstrates that IRE1α/XBP1 axis of ER stress pathways plays a pivotal role in the eye during bacterial (*S. aureus*) endophthalmitis through regulation of innate inflammatory responses. The pharmacological inhibition of this pathway markedly increased disease severity by increasing bacterial proliferation and more retinal tissue damage in the

eye. Most importantly, we found that bacterial-induced IRE1α/XBP1 signaling was TLR2 dependent. Since the attenuation of ER stress has been mostly shown beneficial in eye diseases (54-56), our study reveals what we believe an unconventional role for the IRE1α/XBP1 pathway as a crucial regulator of ocular innate immunity (**Fig 7**).

Our rationale of the current study emerged from an earlier transcriptomics analysis (29), showing induced expression of *Xbp1* and *Bip* transcripts in *S. aureus* infected mouse retina, indicating their role in the pathobiology of bacterial endophthalmitis. Although several ER stress pathways can be triggered in mammalian cells under infectious and inflammatory conditions, we did not observe the activation of ATF6 or the PERK pathway. We therefore, hypothesized that the primary pathway involved in our disease model is the IRE1α-XBP1 pathway. This is supported by multiple pieces of evidence, including, splicing of IRE1α downstream target XBP1 and the attenuation of an inflammatory response and severe disease pathology by pharmacological inhibition of IRE1α. These observations corroborate with studies where activation of IRE1-XBP1 mediated ER stress has also been reported via various intracellular pathogens such as *Chlamydia trachomatis* (57), *Brucella abortus* (58) and *Francisella tularensis* (27). However, some other bacterial species and their toxins such as subtilase toxin produced by *Escherichia coli* (59), and listeriolysin O, produced by *Listeria monocytogenes* (60) have been shown to activate other arms of the UPR as well. In addition to live *S. aureus* infection, we found that TLR2 ligand, Pam3CSK4, and other *S. aureus* virulence factors (PGN, LTA, α-toxin) also activate the IRE1-XBP1 pathway, indicating the potential involvement of TLR2 signaling in regulating an IRE1 mediated ER stress response. Moreover, LPS induced IRE1α activation through TRAF6 has also been reported by Zhang and co-workers in collaboration with our laboratory (61). Consistent with our data, other

studies have suggested that TLR2 can activate the IRE1α pathway, but not the ATF6 or PERK pathways (27).

Furthermore, we established the role of TLR2 signaling in *S. aureus* induced ER stress using TLR2 and its downstream adaptor, MyD88 deficient mice. We found that *S. aureus* induced IRE1α-mediated ER stress is TLR2 dependent but only partially dependent on MyD88 pathways. Prior reports have shown impaired XBP1 splicing in TLR2-deficient macrophages following TLR2 agonists Pam3CSK4, FSL1, *F. tularensis* challenge and MRSA infection (27, 47). Similarly, *Xbp1* splicing has been reported in macrophages by both MyD88 dependent and independent mechanisms(27). Both TLR-signaling and IRE1-mediated splicing of *Xbp1* have been shown to invoke innate immune responses and the production of inflammatory cytokines (27, 36, 47, 62). Our data demonstrated that inhibiting IRE1α attenuates *S. aureus* induced cytokine production *in vivo* (mouse model of endophthalmitis) as well as in cultured microglia, implicating its role in regulating the inflammatory innate immune response. The experiments using BMDM from myeloid cell-specific IRE1$^{-/-}$ mice further validates these findings by showing inhibition of *S. aureus* induced inflammatory mediators. Thus, we conclude that TLR2 triggers IRE1α activation to initiate an innate immune response in *S. aureus* endophthalmitis.

Previous studies from our laboratory and other investigators have shown that in ocular infections, NF-κB and MAPK activation are key players in orchestrating inflammatory responses by various retinal cell types (10, 30, 44). In this study, we demonstrated that in response to *S. aureus* infection, IRE1α mediated ER stress controls the activation of NF-κB, and MAPK such ERK, and p38 signaling pathways. Similarly, the inhibition of these pro-

inflammatory signaling pathways by using their pharmacological inhibitors, resulted in impaired splicing of *Xbp1*, suggesting positive feedback inhibition of ER stress. These results indicate that diminished pro-inflammatory cytokines production upon IRE1 inhibition could be due to the downregulation of *S. aureus* induced NF-κB and MAPK activation.

Reactive oxygen species (ROS) production and ER stress induction are a part of a positive feedback loop(47, 63). Innate immune cells also utilize ROS to kill pathogens, including *S. aureus* (9, 47). We previously showed *S. aureus* infection lead to ROS production in retinal cells (9). Our experiment utilizing NADPH oxidase inhibitor, DPI, resulted in reduced *Xbp1* splicing and diminished production of inflammatory cytokine, establishes a link between ROS generation and IRE1α activation (47). Our data showed an increased bacterial burden in the eyes upon IRE1α inhibition, indicating that IRE1α-mediated ER stress contributes towards antibacterial activity. Since we observed reduced PMN infiltration in eyes treated with IRE1α inhibitor, it remains to be elucidated whether the increased bacterial burden is due to reduced ROS generation within PMNs, less PMN recruitment, or a combination of both and warrants further investigation. IRE1α deficiency has been shown to reduce bacterial killing both in *in-vivo* as well as *in-vitro* models (47) resulting in extensive liver damage in *F. tularensis* infection (27). These observations coincide with our findings of elevated bacterial burden with increased retinal tissue damage due to IRE1α inhibition.

In summary, our study demonstrates an essential role of IRE1α-mediated ER stress response in orchestrating the retinal innate immune responses in bacterial endophthalmitis. We also found that an IRE1α-mediated innate immune response is regulated by TLR2, which has been shown to exert a protective effect in *S. aureus* endophthalmitis. These

findings uncover an important mechanism that can modulate ocular inflammation and could, therefore, provide new opportunities for the development of anti-inflammatory therapies and treatments for ocular infections.

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AUTHORS CONTRIBUTIONS

A.K., P.K.S, and A.K. conceived the project and designed the experiments; A.K. and P.K.S. performed the experiments and analyzed the data; K.Z. and A.K. contributed reagents/ materials/ analysis tools; A.K., P.K.S., and A.K. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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570 **FIGURE LEGENDS**

571 **Figure 1. *S. aureus* infection induces ER stress in the mouse retina.** C57BL/6 mouse
572 (n=6) eyes were intravitreally injected with PBS (control, C) or 5000 CFU of *S. aureus* (SA),
573 strain RN6390. At indicated time point post-infection, eyes were enucleated, and retinal
574 tissue was subjected to temporal transcriptomic analysis using microarray (reported
575 previously (29). The microarray data showed induced expression of *Xbp1* and *Bip* genes.
576 The data are expressed as relative fold change by normalizing the expression of genes with
577 respect to control **(A)**. In another set of experiments, RNA was extracted from SA-infected
578 retinal tissue at 24h and subjected to RT-PCR to detect mRNA expression of indicated ER
579 stress markers **(B)**. Results are representative of at least two independent (n=6 each)
580 experiments. Statistical analysis was performed using one-way ANOVA **, P* <0.05; **, *P*<
581 0.001. Data are shown as the mean ± SD

582 **Figure 2. *S. aureus* induces IRE1α mediated ER stress via TLR2.** C57BL/6 (WT), TLR2^{-}
583 $^{/-}$, and MyD88$^{-/-}$ mouse (n=6 each) (all on B6 background) eyes were intravitreally injected
584 with 4μ8C (0.1μg/eye), 12h post drug injection, SA endophthalmitis was induced, and retinal
585 tissue (24h post-SA infection) was harvested and subjected to RT-PCR to detect IRE1α
586 expression and XBP1 splicing, using GAPDH as housekeeping gene **(A)**. The protein levels
587 of pIRE1α and IRE1α were assessed by western blot **(B, left panel),** and band intensities
588 were quantified using ImageJ, normalized with β-actin, and represented as a bar graph **(B,**
589 **Right panel)**. Mouse microglial cells (BV2 cell line) were left untreated or pre-treated with
590 IRE1α inhibitor, 4μ8C (100 nM), followed by challenge with *S. aureus* (SA), TLR2 agonist
591 (Pam3CSK4, 10 μg/ml), and, TLR4 agonist (LPS, 10 μg/ml) for 8h. The mRNA expression

of indicated ER stress markers was assessed by RT-PCR **(C)**. The protein levels of pIRE1α and IRE1α were assessed by western blot **(D, left panel),** and band intensities were quantified using ImageJ, normalized with β-actin, and represented as a bar graph **(D, Right panel)** Results are representative of at least three independent experiments. Statistical analysis was performed using one-way ANOVA*, P* <0.05; ***, P* <0.01; ****, P* <0.0001; ns, not significant.

Figure 3. IRE1α regulates *S. aureus* and TLR2 ligand-induced inflammatory response. C57BL/6 (WT) and TLR2$^{-/-}$ mouse (n = 6 per group) eyes were injected with IRE1α inhibitor, 4μ8C (0.1 μg/eye), 12h prior to induction of *S. aureus* endophthalmitis and at 24h post-infection eye lysates were subjected to ELISA for measurements of indicated cytokines/chemokines **(A)**. BV2 microglial cells were pre-treated with 4μ8C (100 nM, for 1h) followed by challenge with SA (MOI 10:1), and Pam3CSK4 (10 μg/ml) for 8h. The conditioned media was used for ELISA for the quantification of indicated cytokines/chemokines **(B)**. The production of inflammatory mediators was assessed in IRE1$\alpha^{flox/flox}$, and myeloid cell-specific IRE1α$^{-/-}$ BMDM (MΦ) challenged with SA (MOI 10:1) for 8h using ELISA **(C)**. Results are cumulative of at least two independent (n=6 each) experiments. Statistical analysis was performed using Student's t-test *, *P*<0.05; **, *P*< 0.01; ***, *P*<0.001; ****, *P*<0.0001; ns, not significant. Data are shown as the mean ± SD.

Figure 4. IRE1α inhibition attenuates *S. aureus* induced NF-κB and MAPK signaling. C57BL/6 mouse (n = 6 per group) eyes were injected with IRE1α inhibitor, 4μ8C (0.1 μg/eye), 12h prior to the induction of *S. aureus* (SA) endophthalmitis. At 24h post-infection, retinal tissue lysates were subjected to western blot analysis for pIκB, pERK, and pp38

signaling pathways (**A, Left panel)**. Band intensities were quantified using ImageJ, normalized with β-actin, and represented as fold change in a bar graph **(A, Right panel)**. BV2 cells were pre-treated for 1h with NF-κB, p38, and ERK inhibitors (10 μM each) followed by SA (MOI 10:1) challenge for 8h. Total RNA was extracted, reverse transcribed, and subjected to RT-PCR for XBP1 spliced (XBP1s) and unspliced (XBP1u) forms. Statistical analysis was performed using one-way ANOVA. Data represent mean ± SD. from two independent (n=6 each) experiments **, P* <0.05; ***, P* <0.01; ***, *P*< 0.001.

Figure 5. ROS inhibition reduces *S. aureus* induced XBP1 splicing and inflammatory mediators. BV2 microglial cells were left untreated or pre-treated with NADPH oxidase inhibitor, DPI (1 μM) for 1h followed by *S. aureus* (SA) challenge for 8 h. Total RNA was extracted, reverse transcribed, and subjected to RT-PCR for detection of IRE1α and XBP1 (unspliced: XBPu, spliced: XBPs) **(A)**. ELISA was performed from conditioned media for indicated cytokines/chemokines quantification **(B)**. Results are cumulative of at least three independent experiments. Statistical analysis was performed using Student's t-test, ****, *P*<0.0001. Data are shown as the mean ± SD.

Figure 6. IRE1α inhibition aggravates disease pathology in mouse eyes. Eyes of C57BL/6 WT mouse (n = 6) were pre-treated by intravitreal injection with 4μ8C (0.1 μg/eye), followed by induction of *S. aureus* (SA) endophthalmitis. Twenty-four hours post-SA infection, eyes were enucleated, lysate was prepared in sterile PBS, and the bacterial count was measured by serial plate dilution and represented as CFU per eye **(A)**. Histological analysis was were performed at 24h post-infection by paraffin embedding and H&E staining. 4μ8C alone injected eyes were used as control **(B)**. Retinal cell death was visualized by

TUNEL staining on cryosections showing TUNEL-positive cells (green) and DAPI stained nuclei (blue) **(C).** Flow cytometry was used to assess PMN infiltration by pooling retina from two eyes and staining single-cell suspensions with anti-CD45-PECy5 and anti-Ly6G-FITC monoclonal antibodies **(D)**. Representative dot plots show the percentage of dually positive PMNs (upper right quadrants in **upper panel**). The bar graph shows cumulative quantitative data from three independent experiments (**lower panel**). (ONH: optic nerve head, RPE: retinal pigmented epithelium layer, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer, VC: vitreous chamber, L: lens) Statistical analysis was performed using Student's t-test. Data represent mean ± SD from two independent (n=6 each) experiments ***, P <0.01.

Figure 7. A schematic illustration of IRE1α in regulating the innate inflammatory response in bacterial endophthalmitis. TLR2 recognizes *S. aureus* and induces IRE1α activation through ROS generation. This causes splicing of IRE1α downstream target, XBP1, which activates NF-κB and MAPK signaling resulting in the production of pro-inflammatory cytokines/chemokines. *S. aureus* infection primarily induces, IRE1α-XBP1 axis of the ER stress response. The schematic diagram was created using BioRender software.