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**TLR2 Mediates the Innate Response of Retinal Muller Glia to Staphylococcus aureus**

Nazeem Shamsuddin* and Ashok Kumar*†

Muller cells, the principal glia of the retina, play several key roles in normal and various retinal diseases. To date, their direct involvement in retinal innate defense against bacterial pathogens has not been investigated. In this article, we show that Muller cells express TLR2, a key sensor implicated in recognizing Gram-positive bacteria. We found that intravitreal injection of TLR2 agonist Pam3Cys and *Staphylococcus aureus* activated Muller glia in C57BL/6 mouse retina. Similarly, Pam3Cys or *S. aureus* elicited the expression of TLR2 and activated the NF-κB and p38 MAPK signaling cascade. Concomitant with the activation of signaling pathways, transcriptional expression and secretion of various proinflammatory cytokines (IL-6, TNF-α, and IL-1β), chemokines (IL-8), and antimicrobial peptide (LL-37) were also induced in Muller glia. Importantly, the culture media derived from TLR2-activated Muller glia exhibited robust bactericidal activity against *S. aureus*. Furthermore, use of neutralizing Ab, small interfering RNA, and pharmacological inhibitors revealed that Muller glial innate response to *S. aureus* is mediated via the TLR2–NF-κB axis. Collectively, this study for the first time, to our knowledge, establishes that the retinal Muller glia senses pathogens via TLR2 and contributes directly to retinal innate defense via production of inflammatory mediators and antimicrobial peptides. *The Journal of Immunology*, 2011, 186: 7089–7097.

*Staphylococcus* are a major constituent of extracellular flora, and they often gain access to the intraocular compartments due to trauma or ocular surgery, leading to the development of endophthalmitis (1). Among staphylococci, *Staphylococcus aureus* causes severe endophthalmitis, resulting in greatly diminished or complete loss of visual acuity, despite therapeutic intervention (2). The overall incidence of endophthalmitis has been reported to be between 0.056 and 1.3% after cataract surgery (3–5) and as high as 30% after trauma in a rural setting (6). Because staphylococci are the primary organisms associated with postoperative endophthalmitis and the potential cause for severe vision loss, animal models of experimental staphylococcal endophthalmitis have been developed to investigate the pathogenesis and treatment of this disease (7, 8).

The host–pathogen interaction in the retina has been the subject of extensive research for the last several years (7), and studies have been performed to define the role of various bacterial virulence factors (toxins, cell wall components) in the pathogenesis of endophthalmitis (9). In contrast, very few studies have investigated the host response in this disease (2, 10). This is probably because classically, the retina has been viewed as an immune-privileged tissue (i.e., when infectious organisms or tumor cells are placed into the eye); the destructive immune response is attenuated, thus allowing for the preservation of vision (11). However, recent new approaches and models have led to the increased understanding of the mechanisms of ocular inflammation and innate immunity that are operative in the abrogation of immune privilege postinfection (12). The major protective mechanism that controls the infiltration of inflammatory cells and macromolecules into the posterior segment of the eye is the blood–retina barrier (BRB) (13). Because the production of proinflammatory cytokines and chemokines has been shown to contribute to BRB dysfunction in uveitis (14, 15), the increased BRB permeability in endophthalmitis may also be a consequence of pathogen-induced production of proinflammatory mediators (16). Thus, when BRB function is compromised, it will allow the infiltration of immune cells into the retina, resulting in massive inflammation as seen in patients with infectious endophthalmitis (17). This inflammation, if not controlled, could lead to tissue destruction and vision loss (16). How pathogens are recognized and inflammation is initiated in the retina are still not well defined. The retina, being a part of the CNS, must employ its resident glial cells (microglia, Muller glia, or astroglia) for initial recognition and response to invading pathogens. We hypothesized that retinal cells use TLRs for early detection and initiation of innate responses and recently showed that TLR2 is an important component in providing retinal innate defense against *S. aureus* by demonstrating that pretreatment of the mice with TLR2 ligands prevented the development of staphylococcal endophthalmitis (18).

Because we have found that multiple cells (microglia, Muller glia, and retinal pigment epithelium) in the retina express TLR2 (18), they should be capable of responding toward *S. aureus*. To date, the relative contribution of each cell type in retinal innate defense is not known. Muller cells are the most abundant glial cell type in the retina. They span the entire thickness of the retina and have secondary processes that closely wrap around neuronal cell bodies and dendrites. Muller cell gliosis has been proposed to be neuroprotective in the early stages after retinal injury, perhaps reflecting a cellular response to protect the tissue from further damage (19, 20). Muller cells have also been shown to act as
modulators of immune and inflammatory responses by producing proinflammatory cytokines (21). General signs of Muller cell activation are cellular hypertrophy, upregulation of glial fibrillary acidic protein (GFAP), and the intermediate filament vimentin (22). Although some studies have shown that GFAP is increased in the retina during bacterial endophthalmitis (23) and viral retinitis (24), no study has reported hitherto the direct involvement of Muller glia in pathogen recognition. Thus, the current study was aimed to assess the innate responses of Muller glia to *S. aureus* or a specific TLR2 ligand. Furthermore, we investigated the effect of TLR2 activation on the innate defense against bacterial growth. Our findings demonstrate an active role of the retinal Muller glia against bacterial infection and that TLR2-activated Muller glia-derived factors possess strong bacterialic properties.

Materials and Methods

**Bacterial strain and reagents**

*S. aureus* strains RN 6390 and NCTC 8325 were maintained in tryptic soya broth (Sigma-Aldrich, St. Louis, MO). Bacterial lipopeptide Pam3Cys-Ser-Lys (Lys), hydrochloride (Pam3Cys), a synthetic lipopeptide that acts as an exclusive TLR2 agonist, was purchased from Invivogen (San Diego, CA). The NF-κB inhibitor isohelenin and p38 MAPK inhibitor SB203580 were purchased from Calbiochem (Gibbstown, NJ). A mouse TLR2-neutralizing mAb (clone 2392) was a gift from Invivogen (San Diego, CA). Anti–TLR2, and HSP-90 Abs were purchased from Cell Signaling Technology (Beverly, MA). A mouse monoclonal anti–p-actin Ab was purchased from Santa Cruz Biotechnology, Santa Cruz, CA (1:20 dilution) and were incubated overnight at 4°C. Cells were washed and incubated with the corresponding secondary FITC-conjugated Ab (Invitrogen) (1:200 dilution) for 30 min. A BD LSR II flow cytometer (Immunochemistry Systems; BD Biosciences, San Jose, CA) was used for cytometric analysis.

**Western and dot blot analysis**

MIO-M1 cells lysates were prepared with radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris·HCl [pH 7.5], 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktails [Sigma-Aldrich], and 0.1 mM PMSF), and protein concentration was determined using the BCA assay (micro BCA; Pierce, Rockford, IL). Proteins (30–40 μg/well) were separated by SDS-PAGE in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) and electrobotted onto nitrocellulose transfer membranes (Bio-Rad). After blocking for 1 to 2 h in PBS with Tween-20 (PBST) (20 mM Tris·HCl, 150 mM NaCl, and 0.5% Tween) containing 5% nonfat milk, the blots were probed overnight at 4°C with desired Abs as described by the manufacturers (Cell Signaling Technology, Sigma-Aldrich). NF-κB activation was determined in terms of inhibitory IκB phosphorylation and degradation using anti-IκBα and anti-phospho-IκBα Abs. After washing three times in PBST, membranes were incubated with secondary HRP-conjugated anti-mouse or anti-rabbit IgG Abs (Bio-Rad) for 1 h. After washing with PBST four times for 10 min each, proteins were visualized with Super Signal reagents from Pierce.

Accumulation of LL-37 in the culture media of TLR2 ligand and bacteria-treated MIO-M1 cells was detected by immunoblotting as described previously (26). Briefly, culture media were collected posttreatment, centrifuged, and 100 μl was applied to a nitrocellulose membrane (0.2 μm; Bio-Rad) by vacuum using a dot blot apparatus (Bio-Rad). The membrane was fixed by incubating with 10% formalin for 2 h at room temperature followed by blocking in TBS containing 5% nonfat powdered milk for 1 h at room temperature. The membrane was then incubated overnight at 4°C with rabbit anti-LL-37 diluted 1:4000 in TBS containing 5% nonfat powdered milk, 5% goat serum, 0.05% Tween-20, and 0.02% sodium azide. After washing, the membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to HRP diluted 1:2000 with 5% nonfat powdered milk. Immunoreactivity was visualized with Super Signal reagents from Pierce.

**ELISA for cytokine analysis**

MIO-M1 cells were plated (5 × 10^5 cells per well) in six-well plates. After growth factor starvation, cells were challenged with Pam3Cys or live/heat-killed *S. aureus* (HKSVA) for various times, and culture media were collected for measurement of human TNF-α, IL-1β, IL-6, and IL-8 by ELISA. The cells were lysed, and protein concentration was determined. In some experiments prior to stimulation, MIO-M1 cells were pretreated with TLR2-neutralizing Ab or NF-κB/P-38 MAPK pathway inhibitors as described above. ELISAs were performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The amounts of cytokines in cell culture media were expressed as picograms per milligram of cell lysate. All values were expressed as mean ± SD. Statistical analysis was performed using the Student *t* test.

**Immunofluorescence staining**

Retinal cryosections were rinsed in PBS and blocked for 1 h in blocking buffer (10% [v/v] normal goat serum, 0.3% [v/v] Triton X-100 in PBS) at room temperature. The slides were then incubated overnight with anti-GFAP (DakoCytomation) or anti-vimentin (Sigma-Aldrich) Ab at 4°C. After removal of the primary Abs, slides were extensively washed and incubated for 1 h in fluorescent-conjugated secondary Abs (FITC) at room temperature. After several washing steps in PBS, the slides were mounted in Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA) and visualized using a confocal system (Leica Microsystems, Wetzlar, Germany).

For in vitro staining, MIO-M1 cells cultured on glass chamber slides (Fisher Scientific, Rochester, NY) were stimulated with Pam3Cys or live/heat-killed bacteria for indicated time periods. Cells were washed three times with PBS and then fixed for 15 min in PBS with 4% paraformaldehyde. After washing with gentle shaking, cells were permeabilized for 5 min with ice-cold methanol and washed. The fixed cells were then blocked in 5% [v/v] serum for 1 h at room temperature followed by incubation with TLR2 (Santa Cruz Biotechnology) (1:200 dilution), IL-37 (Imgenex) (1:100 dilution), and vimentin (Sigma-Aldrich) (1:200 dilution) Abs overnight at 4°C. In control experiments, cells were incubated with nonimmune IgG as isotype controls (Santa Cruz Biotechnology; Imgenex, San Diego, CA). Following removal of the primary Abs, the cells were incubated with or without ligands were dispensed in Eppendorf tubes and were centrifuged at 100 × g at 4°C for 5 min. Cells were then resuspended in PBS containing 1% BSA. The cells were blocked in 10% serum for 30 min at room temperature. The cells were then washed, resuspended in PBS containing anti-TLR2 Ab or isotype-matched IgG (Santa Cruz Biotechnology, Santa Cruz, CA) (1:20 dilution) and were incubated overnight at 4°C. Cells were washed and incubated with the corresponding secondary FITC-conjugated Ab (Invitrogen) (1:200 dilution) for 30 min. A BD LSR II flow cytometer (Immunochemistry Systems; BD Biosciences, San Jose, CA) was used for cytometric analysis.
were then extensively washed with PBS and incubated for 1 h with specific FITC-conjugated secondary Abs (1:200 dilution) at room temperature. Finally, the cells were washed extensively with PBS, and the slides were mounted in Vectashield antifade mounting medium (Vector Laboratories) and visualized using a confocal system (Leica Microsystems).

Zone of inhibition assay

The antibacterial activity of conditioned media derived from control or TLR2-activated MIO-M1 cells was tested against *S. aureus* strains (RN 6390 and NCTC 8325) by measuring the zone of inhibition using the disc diffusion method (27). Briefly, bacterial inoculum was prepared from both cultures, and ~1 × 10⁶ CFU/ml were applied as a lawn using plate spreader. The sterile discs soaked with 10 μl culture media derived from 1 million MIO-M1 cells grown in 1 ml media were placed on the surface of bacterial lawn culture. Gentamicin (10 μg/disc) was used as standard antibiotic positive control. The plates were incubated overnight at 37°C, and diameters of inhibition zones were measured. The p values were calculated using the Student t test.

Results

*S. aureus* and TLR2 ligand activate Muller glia in C57BL/6 mouse retina

GFAP-positive staining in Muller cells has been used as a reliable indicator of acute and chronic retinal pathology (19). Under normal conditions, Muller glia express GFAP only in their endfeet. But when pathologic events occur, they alter their characteristics to become reactive glial cells and express GFAP in cell bodies and processes. To assess whether TLR2 activation influences GFAP levels in vivo, mice were given intravitreal injection of a TLR2 ligand, Pam3Cys, or *S. aureus*. The cell bodies and radially oriented processes (arrows in Fig. 1) of the Muller cells were not stained with GFAP Ab in the control, PBS-injected mice. In contrast, Muller glial processes became GFAP positive in mice challenged with Pam3Cys or *S. aureus*. Moreover, the response was concentration dependent, as increasing Pam3Cys dosage increased GFAP expression accordingly in Muller glia processes.

*S. aureus* and Pam3Cys induce TLR2 expression in Muller glia

TLR2 along with TLR1 and TLR6 plays a key role in innate defense against Gram-positive bacteria. To determine whether Muller glia expresses these receptors, RT-PCR analysis was performed using the human retinal Muller glia cell line, MIO-M1. As shown in the Fig. 2A, compared with the control (untreated) cells, the expression of TLR2 mRNA was significantly increased in both Pam3Cys and *S. aureus*-stimulated cells at 4 h and remained elevated up to 24 h (data not shown). In contrast, the expression levels of TLR1 and TLR6 did not change during the course of the study. We also performed the semiquantitative densitometric analysis by normalizing induced TLR2 mRNA to internal control GAPDH mRNA. Clearly, stimulation of MIO-M1 cells by both *S. aureus* and Pam3Cys for 4 h upregulated TLR2 expression by >2.5-fold (Fig. 2B). The induced expression of TLR2 at the protein level was detected by Western blotting using an anti-TLR2 Ab (Fig. 2C); the exposure of MIO-M1 cells to *S. aureus* or Pam3Cys for 4 and 8 h resulted in an increase in the levels of TLR2. To provide further evidence of induced TLR2 expression, we also performed immunohistochemistry (Fig. 2C) and flow cytometry (Fig. 2D) on MIO-M1 cells, which showed that both Pam3Cys and *S. aureus* stimulation augmented TLR2 expression.

*S. aureus* and Pam3Cys induce NF-κB and MAPK signaling in Muller glia

The expression of TLR2 in Muller glia cells suggests that these cells would be responsive to TLR2 agonist-mediated signaling. To test this hypothesis, we measured the ability of Pam3Cys and *S. aureus* to activate NF-κB and MAPK (Fig. 3). First, we challenged the MIO-M1 cells with increasing concentrations of Pam3Cys and showed that these cells were responsive to Pam3Cys at levels as low as 0.01 μg/ml as measured by IkBα phosphorylation, an indicator of NF-κB activation. Treatment of MIO-M1 cells with 1 or 10 μg/ml Pam3Cys resulted in the maximum phosphorylation of IkBα (Fig. 3A). A similar concentration-dependent trend was observed on the phosphorylation of p38, a member of the MAPK family.

Time-course studies of the Muller glia response to Pam3Cys (Fig. 3B) and *S. aureus* (Fig. 3C) were also performed. MIO-M1 cells stimulated with 10 μg/ml Pam3Cys resulted in IkBα phosphorylation detectable at 15 min, and its levels remained elevated at 90 min poststimulation. Accompanying the increase in IkBα phosphorylation, IkBα-degradation was observed 30 min post-stimulation and was maximal at 90 min (Fig. 3B). Time-dependent IkBα phosphorylation and degradation was also observed in MIO-M1 cells challenged with live *S. aureus* (Fig. 3C). The time course of p38 phosphorylation was similar to that of IkBα, with maximal activation detected 90 min after stimulation. Together, these findings demonstrate that TLR2 stimulation of Muller glia induces phosphorylation of IkBα and MAPKs within a similar time frame.

*Muller glia produces inflammatory mediators in response to S. aureus and Pam3Cys challenge*

To assess the biological relevance of induced NF-κB and p38 MAPK signaling, we determined the effects of *S. aureus* and Pam3Cys on the expression and secretion of proinflammatory cytokines using RT-PCR. Compared to the controls (Fig. 4, lane C, Table I), both Pam3Cys and *S. aureus* induced the expression of IL-6, IL-8, TNF-α, and IL-1β in a time-dependent manner (Fig. 4). The mRNA levels of GAPDH (internal control) remained largely unchanged in both control and stimulated cells (Fig. 4). The protein levels of induced cytokines were assessed by ELISA. Significantly increased amounts of IL-6, IL-8, TNF-α, and IL-1β accumulated in the culture media (Fig. 5) of MIO-M1 cells challenged with Pam3Cys and live or HKSA. The response is time dependent and compared with Pam3Cys, *S. aureus* (live and heat-killed) appeared to be a stronger inducer of cytokine secretion in MIO-M1 cells. Taken together, these results indicate that the Muller glia is responsive to TLR2 agonists via the expression and production of proinflammatory cytokines and chemokines.

*S. aureus*-induced inflammatory response in Muller glia is mediated by TLR2 and NF-κB

Having shown that both Pam3Cys and *S. aureus* activated downstream signaling pathways and induced the production of
inflammatory mediators, we next determined whether TLR2 is required for this response. In the presence of functional blocking Abs against TLR2, the production of IL-8 was reduced 62% and 82% in cells challenged with live *S. aureus* (Fig. 6A) or HKSA (Fig. 6B), respectively. In addition to Ab-mediated inhibition of TLR2 signaling, we also used the siRNA approach to knock down TLR2 expression. MIO-M1 cells were transfected with TLR2 siRNA or nontargeted (scrambled) siRNA control and then challenged with *S. aureus* or HKSA for 8 h. As shown in Fig. 6C, the siRNA specifically knocked down the TLR2 expression with no effect on the mRNA levels of the housekeeping gene GAPDH. Consistent with reduced TLR2 expression, siRNA-transfected cells secreted significantly reduced levels of IL-8 compared with nontransfected or scrambled siRNA control (Fig. 6A, 6B). A similar trend was observed for the levels of IL-6 (Fig. 6C, 6D). These results indicate that functional blocking of TLR2 inhibits *S. aureus*-induced production of inflammatory mediators by Muller glia.

TLR2 engagement leads to the activation of both NF-κB and p38 MAPK signaling pathways (Fig. 3). To evaluate the relative contribution of NF-κB and MAPK signaling, MIO-M1 cells were pretreated with the NF-κB (isohelenin) or p38 MAPK (SB203580) inhibitors followed by *S. aureus* challenge. NF-κB inhibitor effectively blocked both live *S. aureus* and HKSA-induced production of IL-8 by 85% and 92%, respectively. In contrast, p38 MAPK inhibitor partially attenuated IL-8 secretion by 60% and 70% in Muller glia challenged with live *S. aureus* (Fig. 6A) or HKSA, respectively (Fig. 6B). These results confirm the roles of NF-κB and p38 in *S. aureus*-induced inflammatory response in Muller glia.

**TLR2 activation induces the expression of the antimicrobial peptide LL-37 in Muller glia**

Besides inducing proinflammatory mediator release, another consequence of TLR activation is the production of antimicrobial peptides (AMPs) (18). Thus, we next sought to determine the expression and production of LL-37 in TLR2-activated Muller glia. As shown in Fig. 7A, both Pam3Cys and *S. aureus* challenge resulted in a time-dependent expression of LL-37 mRNA detected...
by RT-PCR. Consistent with upregulation of LL-37 mRNA, the dot blot assay revealed that the secreted (active) form of LL-37 accumulated in the culture media of stimulated MIO-M1 cells in a time-dependent manner (Fig. 7B). *S. aureus* induced a rapid (within 1 h) increase in LL-37 peptide, whereas Pam3 stimulation increased levels of LL-37 after 4 h. To further ascertain the expression of LL-37, we performed immunohistochemistry of cultured Muller glia using an anti–LL-37 Ab. Consistently, our immunofluorescence data also show the expression and upregulation of LL-37 in Muller glia (Fig. 7C).

**Conditioned medium of TLR2-activated Muller glia possesses bactericidal activity**

Because Pam3Cys and *S. aureus*-challenged Muller glia produce AMPs, we speculated that TLR2-activated Muller glia would exhibit more inherent antibacterial activity than unstimulated cells. To test the bactericidal activity of Muller glia-conditioned media, we used a qualitative zone of inhibition assay (28). The conditioned media derived from Pam3Cys, HKSA, or *S. aureus*-treated MIO-M1 cells significantly inhibited the growth of two *S. aureus* strains NCTC 8325 and RN 6390 (Fig. 8). Importantly, stimulation with Pam3Cys exhibited the strongest antistaphylococcal activity, followed by HKSA and live *S. aureus*.

### Discussion

Muller cells constitute a functional link between neurons and blood vessels and are responsible for maintaining the homeostasis of the retinal extracellular milieu (29, 30). Despite the important role of Muller glia in various retinal diseases (31), their role in bacterial endophthalmitis is largely unknown. In this study, for the first time, to our knowledge, we report that Muller glia actively participate in retinal innate defense against bacterial pathogens via the action of TLRs. We demonstrated that *S. aureus* and TLR2 ligand challenge upregulate TLR2 expression in Muller glia. Moreover, exposure of Muller glia to *S. aureus* or Pam3Cys resulted in the activation of NF-κB and p38 MAPK signaling pathways and an increased expression and secretion of various proinflammatory cytokines and chemokines (IL-6, IL-8, TNF-α,

### Table I. Sequences and product sizes of PCR primers

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F. forward; R. reverse.

**FIGURE 5.** *S. aureus* and Pam3Cys induced cytokine secretion in Muller glia. MIO-M1 cells were stimulated with Pam3Cys (10 μg/ml), live *S. aureus* (SA), or HKSA for indicated time points. Culture supernatants were used to quantitate indicated cytokines/chemokines accumulation by ELISA, and cells were lysed to determine protein concentration. The levels of cytokines are expressed in picograms per milligrams of cell lysate, and the results represent data (mean ± SD) from three independent experiments performed in triplicate. Statistical analysis was performed using the Student t test, and indicated statistical differences are comparisons of stimulated versus unstimulated control (C). *p < 0.05, **p < 0.001.
and IL-1β). To better understand the involvement of TLRs in the Muller glial innate immune response, we used TLR2-neutralizing Ab and siRNA approaches and showed that the Muller glial innate response to *S. aureus* is TLR2 dependent. The novel finding in this study is that TLR2-activated Muller glia produced the AMP LL-37, and their conditioned media exhibited profound bactericidal activity against *S. aureus*. To the best of our knowledge, this is the first study to demonstrate this phenomenon.

Three types of glial cells (Muller, astrocytes, and microglia) are found in the retina. Compared to other cell types, Muller cells are specialized radial glial cells that span the entire thickness of the retina and contact/ensheath all retinal neurons and capillaries (32). Because the Muller cell endfeet (Fig. 1) lie in the inner limiting membrane (33) next to the vitreous and in close proximity to invading pathogens, we reasoned that Muller glia should possess the ability to sense and quickly respond to infectious stimuli. Our data demonstrated that intravitreal injection of Pam3Cys- or *S. aureus*-enhanced GFAP expression in the mouse retina, an indicative of the responsiveness of Muller glia in vivo. However, it should be noted that retinal astrocytes also express GFAP. Thus, the abundant GFAP immunoreactivity, as seen in the ganglion cell layer, may also be contributed by astrocytes, due to the intertwining of astrocyte processes with the endfeet of the Muller cells (Fig. 1). Although the radial pattern of GFAP immunoreactivity is suggestive of retinal Muller cells, to confirm their identity, we also performed additional immunohistochemical studies using an antivimentin Ab and found that GFAP colocalizes with vimentin (data not shown). Furthermore, using cultured Muller glial cells, the major finding of our study is that these cells recognize and respond to *S. aureus* by expressing and secreting proinflammatory cytokines/chemokines and the cathelicidin (LL-37) AMP. LL-37 may eliminate the invading bacteria through their direct antimicrobial action; the cytokines released by the Muller glia may recruit immune cells to the retina. Hence, our findings suggest an active role of Muller glia in innate defense against bacterial infection in the retina.

*S. aureus* produces a variety of virulence factors that are either cell wall-associated molecules or secreted bacterial proteins (toxins), and their coordinated action leads to the manifestation of staphylococcal infections (34). Previous studies by Booth et al. (35, 36) showed the importance of the toxins in the pathogenesis of staphylococcal endophthalmitis. Because the toxins are produced during the stationary phase of bacterial growth, the early innate immune response must be initiated by cell wall-associated components (37). Consistent with these studies, our data showed that both Pam3Cys (a synthetic bacterial lipopeptide) and HKSA induced an inflammatory response in Muller glia, indicating the role of cell wall components. How do retinal cells recognize these

![FIGURE 6. *S. aureus*-induced inflammatory response is attenuated by inhibition of TLR2 and NF-κB signaling. MIO-M1 cells were pretreated for 1 h with isotype (10 μg/ml), anti-TLR2 (10 μg/ml), p38 MAPK (5 μM) and NF-κB (25 μM) pathway inhibitors followed by challenge with live *S. aureus* (SA; A, C) or HKSA (B, D). After 8 h of stimulation, culture supernatants were collected, and IL-8 and IL-6 levels were quantitated by ELISA. For siRNA transfection, MIO-M1 cells were transfected with nontargeted siRNA (NT-siRNA) or TLR2-siRNA for 48 h, followed by challenge with SA or HKSA for 8 h. TLR2 knockdown was assessed by RT-PCR (E). Data are means ± SD of triplicate cultures and representative of three independent experiments. Statistical analysis was performed using the Student t test, and indicated statistical differences are comparisons of isotype versus TLR2 Ab, NT-siRNA versus TLR2-siRNA, and untreated versus inhibitor treated cells: *p < 0.05, **p < 0.001.](http://www.jimmunol.org/doi/full/10.4049/jimmunol.1700112)
bacterial cell wall components? Based on previous studies from our laboratory (26, 38, 39) and others (40–42), we postulated that TLR2 may be a major receptor involved in responses to Gram-positive bacterial infections and demonstrated that Muller glia express TLR2 and its coreceptors TLR1 and -6. Considering the fact that the retina is usually not exposed to the outside environment, one might expect TLRs to be expressed at basal levels in normal retinal cells, and their activation will result in the upregulation of the same or different TLRs. Indeed, we observed upregulation of TLR2 expression in Muller glia following *S. aureus* and TLR2 agonist stimulation. This increased TLR2 expression may facilitate more efficient pathogen recognition and rapid initiation of the innate immune response. Thus, our data suggest that TLR2 expression by Muller glia may be involved in the host defense against *S. aureus* infection.

TLR2 is an important receptor for the recognition of staphylococcal cell wall components. However, based on the observation that astrocytes or macrophages from TLR2-deficient mice stimulated by *S. aureus* still produce proinflammatory mediators (42, 43), it was suggested that other receptors might also be involved in *S. aureus* recognition. In this study, we showed that treatment of Muller glia with either TLR2-neutralizing Ab or siRNA knockdown of TLR2 almost completely (80–90%) abrogated the production of inflammatory cytokines induced by HKSA- and Pam3Cys-stimulated MIO-M1 cells (data not shown) but not live *S. aureus*-challenged cells (Fig. 6), suggesting the involvement of other receptors for live *S. aureus*. One potential candidate receptor could be nucleotide-binding oligomerization domain (Nod)-like receptors, specifically Nod2, which has been shown to regulate the innate immune response against bacterial pathogens in a TLR-independent manner (44). Moreover, studies have reported the expression of Nod2 in ocular tissues (45) and its role in uveitis (46, 47). Because TLRs are involved in the recognition of pathogens in the extracellular compartment, whereas Nod-like receptors detect the microbial ligands intracellularly (48), we propose that TLR2 is the major receptor for detection of *S. aureus* in Muller glia, and the early innate response is generated by this receptor. Whether Nod2 plays a role in the Muller glial innate response toward *S. aureus* warrants further investigation.

The outcome of host–pathogen interactions frequently leads to the release of proinflammatory mediators that play a key role in the recruitment of immune cells to control infection. In addition to the inflammatory response, TLR activation also leads to the production of AMPs. The two best-characterized families of AMPs are defensins (49) and cathelicidins (50, 51). Cathelicidins are constitutively expressed at high levels by neutrophils (52), and their expression at the mucosal surfaces is induced in response to bacterial infections (53, 54). In the current study, we showed that stimulation of Muller glia by *S. aureus* and the TLR2 ligand significantly upregulated the expression of LL-37 mRNA and protein. Furthermore, detection of the LL-37 (the active secreted form) in the conditioned media of TLR2-activated Muller glia suggests that the mechanism for processing cathelicidin to LL-37 peptide is also activated in Muller glia. This corroborates the results we observed in our recent study whereby intravitreal...