S-layer Impacts the Virulence of *Bacillus* in Endophthalmitis

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Submitted: June 22, 2019
Accepted: July 19, 2019

Citation: Mursalin MH, Coburn PS, Livingston E, et al. S-layer impacts the virulence of *Bacillus* in endophthalmitis. *Invest Ophthalmol Vis Sci*. 2019;60:3727–3739. https://doi.org/10.1167/iovs.19-27453

Purpose. *Bacillus* causes a sight-threating infection of the posterior segment of the eye. The robust intraocular inflammatory response in this disease is likely activated via host innate receptor interactions with components of the *Bacillus* cell envelope. S-layer proteins (SLPs) of some Gram-positive pathogens contribute to the pathogenesis of certain infections. The potential contributions of SLPs in eye infection pathogenesis have not been considered. Here, we explored the role of a *Bacillus* SLP (SlpA) in endophthalmitis pathogenesis.

Methods. The phenotypes and infectivity of wild-type (WT) and S-layer deficient (Δ*slpA*) *Bacillus thuringiensis* were compared. Experimental endophthalmitis was induced in C57BL/6J mice by intravitreally injecting 100-CFU WT or Δ*slpA B. thuringiensis*. Infected eyes were analyzed by bacterial counts, retinal function analysis, histology, and inflammatory cell influx. SLP-induced inflammation was also analyzed in vitro. Muller cells (MIO-M1) were treated with purified SLP. Nuclear factor-κB (NF-κB) DNA binding was measured by ELISA and expression of proinflammatory mediators from Muller cells was measured by RT-qPCR.

Results. Tested phenotypes of WT and Δ*slpA B. thuringiensis* were similar, with the exception of absence of the S-layer in the Δ*slpA* mutant. Intraocular growth of WT and Δ*slpA B. thuringiensis* was also similar. However, eyes infected with the Δ*slpA* mutant had significantly reduced inflammatory cell influx, less inflammatory damage to the eyes, and significant retention of retinal function compared with WT-infected eyes. SLP was also a potent stimulator of the NF-κB pathway and induced the expression of proinflammatory mediators (IL6, TNFα, CCL2, and CXCL-1) in human retinal Muller cells.

Conclusions. Taken together, our results suggest that SlpA contributes to the pathogenesis of *Bacillus* endophthalmitis, potentially by triggering innate inflammatory pathways in the retina.

Keywords: endophthalmitis, bacteria, Bacillus, inflammation, retina, blindness

Severe inflammation and rapid vision loss are destructive consequences of *Bacillus* endophthalmitis.[1–3] This disease typically results from a traumatic ocular injury with a foreign body contaminated with this organism. *Bacillus* endophthalmitis is particularly devastating, as greater than 70% of patients were reported to have lost significant vision, and 50% of those cases resulted in enucleation of the infected eye.[4–10] Treatment strategies for traumatic ocular injuries include the use of antibiotics, anti-inflammatory drugs, and in severe cases, vitrectomy surgery.[11–17] However, the potentially blinding outcome for *Bacillus* endophthalmitis has been difficult to prevent, emphasizing the importance of identifying unique virulence factors of *Bacillus* that might be targeted in developing better treatment strategies for this disease.

B. cereus and *B. thuringiensis* are two of the most virulent organisms reported to cause bacterial endophthalmitis. These members of the *Bacillus cereus sensu lato* group are Gram-positive, facultative aerobic, spore-forming rods, and are widely distributed in the environment.[18,19] Other than the presence of crystal toxins in *B. thuringiensis*, the genomes and phenotypes of *B. cereus* and *B. thuringiensis* are highly similar and, on a genetic basis together with *B. anthracis*, are considered a single species.[20–22] These organisms express a comparable cohort of virulence factors that have the potential to contribute to disease. During infection, *Bacillus spp*. replicates and expresses harmful toxins and enzymes in the vitreous.[23–27] *Bacillus spp*. also possesses a quorum sensing system (PlcR) that regulates the coordinated synthesis of almost all extracel-

lular virulence factors and is important for intraocular virulence.[27,28]

We reported that an absence of individual *Bacillus* toxins, such as hemolysin BL, phosphatidylinositol-specific phospholipase C (PI-PLC), or phosphatidylcholine-specific phospholipase C (PC-PLC), did not completely eliminate endophthalmitis pathology.[24,25] We also reported delayed evolution of *Bacillus* endophthalmitis in the absence of the PlcR quorum sensing system.[23,27,29,30] In these cases, complete elimination of disease pathology did not occur, suggesting the contribution of other nontoxin bacterial products or perhaps *Bacillus* cell wall components in this disease.

During experimental endophthalmitis, *Bacillus* induces a rapid inflammatory response, which is more aggressive than that of other common pathogens associated with this disease.[2,3,31,32] We reported that these inflammatory responses were mediated, in part, through innate receptors, such as Toll-like receptor 2 (TLR2), TLR4, and their adaptors, myeloid differentiation primary response gene-88 (MyD88), and Toll/interleukin-1 receptor (TIR) domain containing adaptor-inducing interferon-β (TRIF).[33,34] *Bacillus* endophthalmitis in mice deficient in TLR2, TLR4, MyD88, or TRIF was significantly less severe than infections in the eyes of WT mice. We also reported that nonviable *B. cereus* cell walls induced a greater degree of intraocular inflammation than cell walls of other Gram-positive pathogens associated with endophthalmitis,[2] suggesting that this difference in inflammation potential may be attributed to variations in cell envelope constituents.

The *Bacillus* cell envelope varies structurally from other Gram-positive ocular pathogens, such as staphylococci or streptococci.[35–38] The envelopes of *Bacillus* and other Gram-positive organisms have an inner membrane, a thick layer of peptidoglycan (PGN), teichoic acids (TA), and lipoproteins (Lpp), and proteinaceous adhesive appendages called pili.[38–42] Unlike other Gram-positive ocular pathogens, *Bacillus* has peritrichous flagella. *Bacillus* species, including some strains of the *Bacillus cereus sensu lato* group, have a paracrystalline surface layer composed of S-layer proteins (SLPs).[43–45] During infection, this pathogen migrates from the posterior to the anterior segment.[2,23] Nonmotile *Bacillus* were less virulent and a deficiency in swarming movement prevented the pathogen from migrating to the anterior segment, resulting in less severe disease.[23,46,47] Flagella aid this migration throughout the eye, but are weak activators of TLR5.[23,47] Recently, we reported a potential protective role for *Bacillus* pili in the clearance of the pathogen during the early stages of endophthalmitis.[48] The inflammatory capacities of common Gram-positive envelope components (Lpp, PGN, and TA) are well documented,[49–52] but the role of the SLPs in the context of *Bacillus* endophthalmitis has not been addressed.

SLPs are cell surface proteins present in Gram-positive and -negative bacteria, as well as in *Archaea*.[53] A spontaneous self-assembly of one or more SLPs forms a regularly spaced array on the surface of the bacterial cell. This array links with the underlying cell surface through noncovalent forces.[54,55] *Firmicutes* SLPs possess two domains, a conserved anchoring domain composed of three repetitions of approximately 50 residues followed by the crystallization domain. Sequence similarities of crystallization domains from different species are low because there are no universal signature sequences.[56] *Archaea*, such as *Thermoproteus, Methanocorpusculum*, and *Sulfolobus*, possess SLPs as a sole constituent of their cell wall, suggesting its role in determination and maintenance of cell shape and function as a molecular sieve.[57–59] SLPs also contribute to microbial pathogenesis through several mechanisms, such as promoting bacterial adherence to host cells and extracellular matrix components, and biofilm formation.[60–64] Bacterial SLPs also provide protection from complement-mediated killing and phagocytosis.[63,65] SLPs protect microorganisms from harmful environmental changes, such as abrupt changes in pH, radiation, and mechanical and osmotic stresses,[66–70] and safeguard the pathogen from bacteriolytic enzymes or antimicrobial peptides, bacteriophages, and other bacterial predators.[71,72]

Because bacterial SLPs are major cell wall proteins of certain microorganisms and contribute to their pathogenesis, and because the *Bacillus* cell wall is highly inflammogenic, we hypothesized that an SLP of *B. thuringiensis* (SlpA) contributes to the pathogenesis of endophthalmitis. Using a well-characterized experimental mouse model of endophthalmitis, we demonstrated that the absence of SlpA impacted *B. thuringiensis* virulence, significantly blunting the severity of experimental endophthalmitis caused by this pathogen. Further exploration of a role for SlpA in *B. thuringiensis* endophthalmitis may identify a new virulence determinant for this pathogen, potentially paving the way for SLPs as novel therapeutic targets for this blinding disease.

Materials and Methods

Ethics Statement

The in vivo experiments described in these studies involved the use of mice. All animal experiments were performed following the recommendations of the Guide for the Care and Use of Laboratory Animals, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (approved protocols 16-086, 18-079).

Bacterial Strains

B. thuringiensis subsp. galleriae NRRL 4045 (WT) or its isogenic S-layer (SL)-deficient mutant (Δ*slpA*)[73] were used to initiate experimental endophthalmitis in mice, as previously described.[1,30,31,34,47,48,74,75] Phenotypes of WT and Δ*slpA B. thuringiensis* were compared by transmission electron microscopy (TEM), growth curves, hemolytic analysis, gel electrophoresis, cytotoxicity, and motility analysis, as described below.

Transmission Electron Microscopy

WT and Δ*slpA B. thuringiensis* were compared by thin-section TEM. Overnight (18 hour) cultures of WT and Δ*slpA B. thuringiensis* were centrifuged and resuspended in PBS, fixed with 2% paraformaldehyde (EM grade), 2.5% glutaraldehyde (EM grade), in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4°C. Samples were then post-fixed for 90 minutes in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate, and rinsed three times for 5 minutes each in the same buffer. The samples were then dehydrated in a graded ethanol series (50%–100%) for 15 minutes each. Bacteria were then dehydrated twice for 15 minutes each in 100% propylene oxide. Following dehydration, the samples were infiltrated in a graded epon/araldite (EMS) resin/propylene oxide series (1:3, 1:1, 3:1) for 60 and 120 minutes, and overnight, respectively. The following day, samples were further infiltrated with pure resin for 45 and 90 minutes, and overnight. The samples were then infiltrated with resin plus benzyl dimethylamine (BDMA, accelerator) for 4 hours and then embedded in resin plus BDMA and polymerized at 60°C for 48 hours. Ultrathin sections were stained with Sato's lead and saturated uranyl acetate in 50% methanol before viewing on a Hitachi H7600 TEM (Hitachi, Tokyo, Japan). All procedures were performed at

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the Oklahoma Medical Research Foundation Imaging Core, Oklahoma City, Oklahoma, United States.

Bacterial Growth Curves

WT and Δ*slpA B. thuringiensis* strains were cultured for 18 hours at 37°C with aeration in brain heart infusion medium (BHI; VWR, Radnor, PA, USA). Strains were then subcultured in fresh BHI to an approximate concentration of 10^3 CFU/mL and incubated for 18 hours as mentioned above. Every 2 hours, 20-µL aliquots were diluted 10-fold in PBS, plated onto BHI agar plates, and quantified.[25,27,48]

Hemolytic Analysis

WT and Δ*slpA B. thuringiensis* were cultured for 18 hours as mentioned above, then centrifuged at 3000*g* for 15 minutes. Supernatants were then filter sterilized (0.22 µm; Millex-GP, Merck Millipore Ltd., Cork, Ireland) and serially diluted 1:2 in PBS (pH 7.4). Diluted supernatants were incubated 1:1 with 4% (vol/vol) sheep erythrocytes (Rockland Immunochemicals, Pottstown, PA, USA) in a 96-well round bottom plate for 30 minutes at 37°C. The plate was centrifuged at 1892*g* for 10 minutes to remove the unlysed erythrocytes.

The supernatants were carefully transferred into a 96-well, flat-bottom plate and hemoglobin release was measured spectrophotometrically at 490 nm by using a FLUOstar Omega microplate spectrophotometer (BMG Labtech, Cary, NC, USA). Values are expressed as the percent hemolysis relative to a 100% lysis control in which 5% rabbit erythrocytes were lysed in double-distilled H_2O.[23,27,48,76,77] Values represent the mean results ± SEM of two independent experiments. These strains were also incubated on tryptic soy agar (TSA) supplemented with 5% sheep blood (Hardy Diagnostics, Santa Maria, CA, USA) for 18 hours at 37°C, and colony morphology and hemolytic phenotypes were compared.

Motility Analysis

WT and Δ*slpA B. thuringiensis* were inoculated at the center of motility agar (0.75% agar in BHI) plates using a sterile toothpick and incubated for 24 hours at 37°C. Distances of each colony from the center were measured in millimeters.[23,27,46]

Muller Cell Cytotoxicity

Immortalized human Muller cells (MIO-M1; a kind gift from Dr. Astrid Limb, UCL Institute of Ophthalmology, London) were maintained in DMEM/F-12 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO, USA) and 1% Pen Strep (GIBCO) in a humidified 5% CO_2 incubator at 37°C.[78,79]

The cytotoxicity of WT and Δ*slpA B. thuringiensis* on human retinal Muller cells was measured using a Pierce Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 20,000 cells/100 µL were seeded in triplicate wells for overnight incubation. WT and Δ*slpA B. thuringiensis* were cultured for 18 hours and centrifuged at 3000*g* for 15 minutes. Supernatants were then filter sterilized (0.22 µm; Millex-GP, Merck Millipore Ltd.) and added into respective wells. Positive and negative controls were 10 µL lysis buffer (10×) and 10 µL of sterile ultrapure water, respectively. Cytotoxicity (%) was calculated by subtracting the LDH activity of negative control from sample LDH activity, divided by the total LDH activity (positive control − negative control), and multiplied by 100.[29,79]

Purification of *Bacillus* S-layer Protein

WT and Δ*slpA B. thuringiensis* were grown for 18 hours at 37°C in BHI, harvested by centrifugation (at 3000*g* for 15 minutes at 4°C), and washed twice with chilled HyPure cell culture grade water (GE Healthcare Life Science, Logan, UT, USA). Pellets were then resuspended in one-tenth of the initial volume of 3 M guanidine hydrochloride (GHCL; pH 2.5; Sigma-Aldrich Corp.) and incubated for 1 hour at 37°C. The extracted SLP was separated from the cell pellets by centrifugation (18,000*g* for 15 minutes, 4°C). The supernatants were carefully transferred and dialyzed (Pur-A-Lyzer 50-kDa dialysis kit; Sigma-Aldrich Corp.) for 24 hours at 4°C against 2 L of tris/HCL (pH 8.0; Research Products International Corporation, Mt. Prospect, IL, USA) with four exchanges of dialysis buffer to remove the residual GHCL. Protein concentrations were measured with a bicinchoninic acid kit (Sigma-Aldrich Corp.) according to the manufacturer's instructions. Endotoxin level was measured using Pierce LAL chromogenic endotoxin quantitation kit (ThermoFisher Scientific) according to the manufacturer's instructions. Samples were then resuspended in Laemmli buffer, boiled for 10 minutes, loaded onto a 12% PAGE. The gels were fixed and stained with Coomassie brilliant blue.[44,73,80]

Isolation of *Bacillus* Peptidoglycan (PGN)

PGN was prepared by the method described in Langer et al.[81] BHI agar plates were inoculated with 0.1 mL of an overnight culture of WT or Δ*slpA B. thuringiensis* and incubated at 37°C overnight. Lawns were scraped from the plates into 50-mL cold BHI and harvested by centrifugation (5000*g*, 37°C, 10 minutes). Pellets were washed in endotoxin free water (GE Healthcare Life Science), resuspended in 5 mL 8% SDS (Sigma-Aldrich Corp.), and boiled for 30 minutes. Lysed cells were then centrifuged at 25,000*g* for 20 minutes and washed 3× with endotoxin-free water. Washed pellets were resuspended with 40 U of DNase I and 7 U of RNase A (ThermoFisher Scientific) and incubated for 15 minutes at room temperature. To remove the nucleases, samples were resuspended in 4% SDS (Sigma-Aldrich Corp.), boiled for 15 minutes, and washed 3× with endotoxin free water, 1× with 2M NaCl, and 6× with endotoxin-free water. Pellets were then resuspended in endotoxin-free water, boiled for 5 minutes, and centrifuged as above. PGN was then dried, weighed, and resuspended in endotoxin-free water to a concentration of 40 mg/mL. Sterility was tested by spread plating on BHI agar. The level of endotoxin was measured by Pierce LAL chromogenic endotoxin quantitation kit (ThermoFisher Scientific) according to the manufacturer's instructions.

TLR2 Reporter Assay for PGN

HEK-Blue reporter cell lines are designed to measure the stimulation of human TLRs by monitoring the activation of Nuclear factor-κB (NF-κB; Invivogen, San Diego, CA, USA). These cells are transfected with a reporter gene encoding secreted embryonic alkaline phosphatase (SEAP), whose transcriptional activation is under the control of a TLR-inducible gene promoter. HEK-293 cells, which do not normally express TLRs, were co-transfected with an expression plasmid encoding specific TLRs. In this study, we used HEK-Blue hTLR2 (named here hTLR2) for the recognition of TLR2 agonists. hTLR2 cells were routinely cultured (up to 20 passages) in Dulbecco's modified Eagle's medium (DMEM) containing GlutaMAX-I (GIBCO), supplemented with 10% (vol/vol) FBS and HEK-Blue Selection antibiotics (Invivogen) in a humidified 5% CO_2 incubator at 37°C.

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PGN (0.1, 1, 10, and 100 μg/mL) from WT and Δ*slpA B. thuringiensis*, 0.25 ng/mL Pam3Csk4 (TLR2 agonist; positive control for hTLR2), and endotoxin-free water (negative control for hTLR2; GE Healthcare Life Science) were added to wells of a 96-well tissue culture plate. Growth medium from 50% to 80% confluent TLR2 reporter cells was discarded and gently washed with warm PBS (pH 7.4; GIBCO). After detaching the cells with PBS (pH 7.4) a new cell suspension of each cell type was prepared (~50,000/180 μL TLR2) in HEK-Blue detection medium (Invivogen). Each cell (180 μL/well) suspension was immediately added into each well and the plates were incubated for 14 to 16 hours at 37°C in 5% CO_2. Production of SEAP from hTLTR2 cells following PGN exposure was measured using a spectrophotometer at 620 to 655 nm.[82] TLR2 activation was presented as percent of TLR2 activation relative to the positive-control Pam3Csk4.

Mice and Intraocular Infection

All in vivo experiments were performed with C57BL/6J mice purchased from commercially available colonies (Stock No. 000664; Jackson Labs, Bar Harbor, ME, USA). Mice were kept on a 12 hour on/12 hour off light cycle, acclimated to housing conditions for at least 2 weeks to equilibrate their microbiota, and maintained under biosafety level 2 conditions during the experiments. All animals were 8- to 10-weeks old at the time of the experiments. Mice were sedated using a combination of ketamine (85 mg/kg body weight; Ketathesia, Henry Schein Animal Health, Dublin, OH, USA) and xylazine (14 mg/kg body weight; AnaSed; Akorn Inc., Decatur, IL, USA). Experimental endophthalmitis was induced by intravitreally injecting approximately 100 CFU WT or Δ*slpA B. thuringiensis*/0.5 μL BHI into one eye using a sterile glass capillary needle, as previously described.[1,33,34,47,48,74–77,83] The uninjected left eye served as the uninfected control. Electroretinography (ERG) was performed prior to euthanasia by CO_2 inhalation and harvesting for subsequent analysis. As described below, quantitation of intraocular bacterial growth, retinal function, polymorphonuclear leukocyte (PMN) infiltration (myeloperoxidase [MPO] activity), and analysis of ocular architecture (histology) were conducted at different time points postinfection.

Intraocular Bacterial Growth

Intraocular bacteria were quantified as previously described.[1,33,34,47,48,74–77,83] Briefly, infected eyes were harvested from euthanized mice at 0, 2, 6, 8, 10, and 12 hours postinfection. Infected eyes were then homogenized in 400 μL PBS with sterile 1-mm glass beads (BioSpec Products, Inc., Bartlesville, OK, USA). Eye homogenates were then diluted 10-fold in PBS, plated onto BHI agar plates and quantified by track dilution.

Retinal Function Analysis by Electroretinography

ERG was used to quantify retinal function in eyes infected with WT or Δ*slpA B. thuringiensis*, as previously described.[1,33,34,47,48,74–77,83] Scotopic ERGs were performed at 6, 8, 10, and 12 hours postinfection using Espion E2 software (Diagnosys LLC, Lowell, MA, USA). After infection, mice were dark adapted for at least 6 hours. Infected mice were anesthetized as previously described and pupils were dilated with topical phenylephrine (Akorn, Inc.). Two gold wire electrodes were placed on each cornea and reference electrodes were place on the forehead and on the tail. Eyes were then stimulated by five flashes of white light (1200 cd s/m^2) and retinal responses were recorded as A-wave and B-wave amplitudes for infected eyes and compared with the uninfected eyes of the same animal.

Histology

Infected eyes were harvested from euthanized mice at 6, 8, 10, and 12 hours postinfection. Harvested eyes were then incubated in low-alcoholic fixative for 30 minutes and then transferred to 70% ethanol. Eyes were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or tissue Gram stain.[1,33,34,47,48,74–77,83]

Inflammatory Cell Influx

Ocular PMN infiltration was estimated by quantifying MPO using a sandwich ELISA (Hycult Biotech, Plymouth Meeting, PA, USA), as previously described.[1,33,34,47,74] At 6, 8, 10, and 12 hours postinfection, eyes were harvested, placed into PBS-containing proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and homogenized using sterile glass beads (BioSpec Products, Inc.). Eye homogenates were tested with the MPO ELISA. Uninfected eye homogenates served as negative controls. The lower limit of detection for this assay was 2 ng/mL.

NF-κB-DNA Binding Assay

NF-κB-DNA binding following SlpA stimulation of MIO-M1 cells was measured by an NF-κB transcription factor assay (Cayman Chemical Co., Ann Arbor, MI, USA), a nonradioactive, sensitive method for detecting specific NF-κB-DNA binding in nuclear extracts.[84] Briefly, 3×10^6 human Muller cells/well were seeded in a 6-well plate and treated with 10 μg/mL SLP for 15 minutes to 2 hours. Untreated wells were negative controls. Nuclear extracts were prepared at the indicated time points using a chemical nuclear extraction kit (Cayman Chemical Co.) according to the manufacturer's instructions. Briefly, cell pellets were washed with PBS/phosphatase inhibitor solution, swelled with hypotonic buffer, lysed with NP-40, centrifuged at 14,000*g*, and resuspended in complete nuclear extraction buffer. After 30 minutes of incubation, suspensions were centrifuged at 14,000*g* and supernatants containing nuclear proteins were collected. To measure NF-κB-DNA binding, 50 μg of nuclear proteins was added to the wells containing transcription factor buffer and incubated overnight at 4°C. Controls included blank wells (negative) and TNFα-stimulated HeLa cell nuclear extract (positive). NF-κB binding was detected with NF-κB primary antibody and goat anti-rabbit horseradish peroxidase conjugate secondary antibody. Absorbance was measured at 450 nm. Changes in NF-κB-DNA binding for each time point were quantified by calculating the percentage of binding relative to the untreated group.

RNA Isolation and Quantitative PCR

Expression of proinflammatory cytokines (IL-6, TNF-α) and chemokines (CCL2, CXCL-1) from MIO-M1 cells treated with SLP were measured by real-time quantitative PCR. MIO-M1 cells were seeded (2×10^6 cells/well) into a 6-well plate and treated with 10 μg/mL SLP from WT *B. thuringiensis*, 10 μg/mL SLP extract control (SLP fraction from Δ*slpA B. thuringiensis*), or PBS for 10 hours. Untreated cells were the negative control. Total RNA was isolated using a QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany). DNA was removed by using a TURBO DNA-free Kit (Invitrogen, Carlsbad, CA, USA). Using an RNA Clean & Concentrator-5 Kit (Zymo Research, Irvine, CA, USA), 50 μL RNA was purified. The concentration and purity were confirmed on a Nanodrop. All procedures were

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Table. Primer Sequence Used in Quantitative PCR

Gene	Species	Sequence (5′–3′)	Temperature (°C)
GAPDH	Human	ACA TCG CTC AGA CAC CAT G TGT AGT TGA GGT CAA TGA AGG G	55
TNFα	Human	TGC ACT TTG GAG TGA TCG G TCA GCT TGA GGG TTT GCT AC	55
IL-6	Human	CCT TCC CTG CCC CAG TA ATT CGT TCT GAA GAG GTG AGT G	55
CXCL-1	Human	CTC TTC TTC CCT AGG AGC GT TGT TCT TGG GGT GAA TTC CC	55
CCL2 (MCP-1)	Human	AGT GTC CCA AAG AAG CTG TG AAT CCT GAA CCC ACT TCT GC	55

performed following the manufacturer's instructions. Real-time quantitative PCR was performed with the Applied Biosystems 7500, using an iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA) and specific primers (Table) following the manufacturer's instructions.[75] PCR amplification was performed in triplicate and water was used to replace RNA in each run as negative control. Relative gene expression was determined using ΔC_T method using GAPDH as a reference housekeeping gene.[75]

Statistics

GraphPad Prism 7 was used for the statistical analysis (GraphPad Software, Inc., La Jolla, CA, USA). Mann-Whitney *U* test was used for statistical comparisons[48,74,77] unless otherwise specified. *P* values of < 0.05 were considered significant.

Results

Absence of S-layer Does Not Alter Bacterial Growth or Phenotypes

The phenotypes of *B. thuringiensis subsp. galleriae* NRRL 4045 (WT) and its isogenic SlpA-deficient mutant (Δ*slpA*) were compared. Thin-section TEM showed the absence of SL in Δ*slpA B. thuringiensis* and its presence in the WT strain (Fig. 1A). In vitro growth and other virulence phenotypes were also compared. Overnight cultures of each strain were subcultured into fresh BHI and bacteria were quantified every 2 hours for 18 hours. Figure 1B demonstrates that WT and Δ*slpA B. thuringiensis* grew to similar concentrations in BHI at all time points, and both strains reached stationary phase growth at 6 hours. In Figure 1C, hemolytic titers of 18-hour supernatants of WT and Δ*slpA B. thuringiensis* were compared by hemolytic assay. Supernatants of these strains were similarly hemolytic. This finding was confirmed by the presence of similarly sized hemolytic zones on TSA supplemented with 5% sheep blood. Figure 1D depicts the characteristic double zones of hemolysis surrounding each colony of WT and Δ*slpA B. thuringiensis* after 18-hour incubation at 37°C. SLPs from both strains were purified, loaded onto a 12% PAGE. The Coomassie stained gel in Figure 1E depicts the presence of a 91.4-kDa band indicating the existence of SlpA in the WT strain. As expected, this band was absent in the Δ*slpA* strain, confirming our TEM data. We also noted the presence of a 49-kDa flagellar band in both WT and Δ*slpA* strains, similar to what has been previously described.[44,80] We measured the cytotoxicity of WT and Δ*slpA B. thuringiensis* supernatants on human retinal Muller cells, and demonstrated that these strains were similarly cytotoxic (Fig. 1F). We also compared the motility of WT and Δ*slpA B. thuringiensis* on motility agar (0.75% agar in BHI). Figure 1G demonstrates that the distances of the colonies from the center to the edge were similar. To determine whether the absence of SlpA altered the *Bacillus* cell wall composition, we purified PGN from WT and Δ*slpA B. thuringiensis*. PGN yields from WT and Δ*slpA B. thuringiensis* were approximately 7.4% and 8.5%, respectively. Because PGN is a universal TLR2 agonist, we measured TLR2 activation by equal concentrations WT and Δ*slpA* PGN in the hTLR2 reporter assay. Figure 1H demonstrates that PGN from both WT and Δ*slpA* activated TLR2 to a similar degree. Together, these results suggested that the absence of SlpA did not affect the in vitro bacterial growth, hemolytic phenotypes, cytotoxic potential of secreted bacterial products, motility, or cell wall composition. These similarities suggested that in vivo infections with WT and Δ*slpA B. thuringiensis* might be similar.

Absence of S-layer Does Not Significantly Alter Intraocular Bacterial Growth

The intraocular growth of WT and Δ*slpA B. thuringiensis* was analyzed in a well-established model of experimental endophthalmitis in C57BL/6J mice (Fig. 2). Eyes were infected with 108 ± 4 CFU/eye of WT or 96 ± 4 CFU/eye of Δ*slpA B. thuringiensis* ($P = 0.0952$). At 2, 6, 8, 10, and 12 hours, infected eyes were harvested, homogenized, and plated onto BHI agar. Intraocular bacterial loads in eyes infected with WT or Δ*slpA B. thuringiensis* were similar, except at 8 hours postinfection. These results also suggested that intraocular infections with WT and Δ*slpA B. thuringiensis* may be similar.

Retinal Function is Retained in the Absence of S-layer Protein

Analysis of retinal function and the representative waveforms of eyes infected with WT or Δ*slpA B. thuringiensis* is depicted in Figure 3. Infected mice were dark-adapted for at least 6 hours and ERG was performed at 6, 8, 10, and 12 hours postinfection. Retention of retinal function, as demonstrated by A- and B-wave amplitudes in WT and Δ*slpA*-infected eyes, was similar at 6 hours postinfection (Fig. 3A). A-wave function, which represents the function of retinal photoreceptor cells, rapidly decreased in eyes infected with WT *B. thuringiensis* from 6 to 12 hours postinfection, to a retained response of approximately 5% (Fig. 3A). This function in Δ*slpA*-infected eyes was also decreased over time, but was retained to a significantly greater degree compared with that of WT *B. thuringiensis*–infected eyes. B-wave function, which represents the function of bipolar cells, Muller cells, and second order neurons, also rapidly decreased in the WT *B. thuringiensis*–infected eyes from 6 to 12 hours postinfection, to a retained response of approximately 10% (Fig. 3B). This response over the same period of time was also decreased in eyes infected with Δ*slpA B. thuringiensis*, but function was

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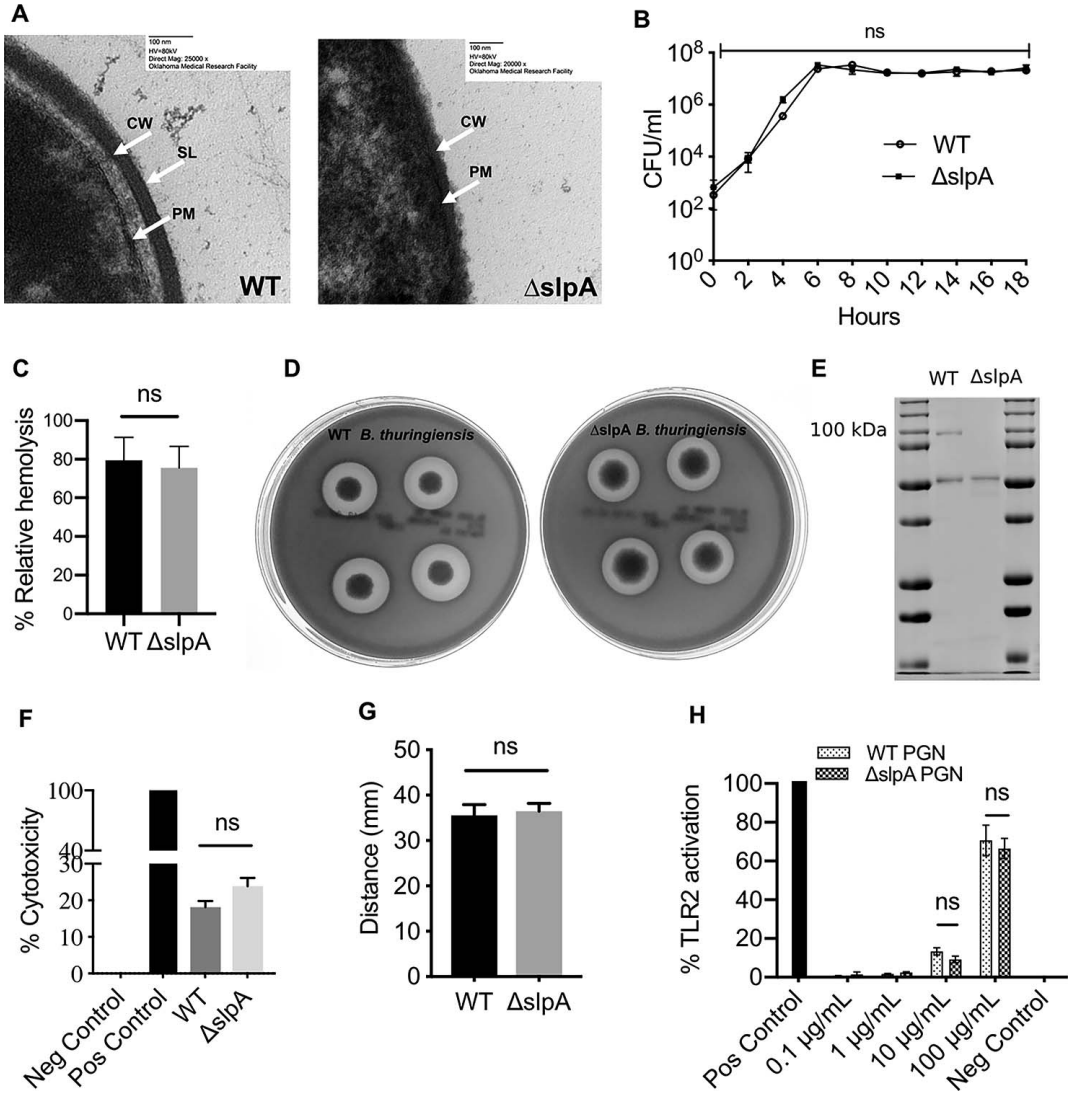

Figure 1. Absence of SLP does not alter bacterial growth and phenotypes. (**A**) Electron micrograph of thin-sections of WT and Δ*slpA B. thuringiensis*. CW, Cell wall; PM, plasma membrane. Magnification, ×25,000 (WT) and ×20,000 (Δ*slpA*). (**B**) In vitro growth curve of WT *B. thuringiensis* and its isogenic SlpA-deficient mutant (Δ*slpA*) in BHI broth. CFU of both WT and Δ*slpA B. thuringiensis* were similar at each time point ($P > 0.05$). Values represent the mean ± SEM for $N = 3$ samples per time point. (**C**) WT and Δ*slpA B. thuringiensis* were compared for their hemolytic activities (2-way ANOVA, $P > 0.05$). (**D**) WT and Δ*slpA B. thuringiensis* exhibited colonies with characteristic double zones of hemolysis on TSA containing 5% sheep blood. (**E**) Samples of the final purification step from WT and Δ*slpA B. thuringiensis* were loaded onto 12% polyacrylamide gel and subjected to PAGE. The 91.4-kDa band indicates the presence of SlpA in the WT *B. thuringiensis*, which is absent in the isogenic mutant. A 49.1-kDa flagellar protein band in both WT and Δ*slpA B. thuringiensis* serves as a standard. (**F**) Cytotoxicity of filter sterilized overnight supernatants from WT and Δ*slpA B. thuringiensis* in human retinal Muller cells. No significant difference was observed in the cytotoxicity of these strains ($P = 0.1297$). Data represents the mean ± SEM of percent of cytotoxicity for $N > 5$ samples. (**G**) The motility of WT and Δ*slpA B. thuringiensis* was compared. Both strains exhibited similar levels of motility ($P = 0.9429$). (**H**) Cell-wall PGN from WT and Δ*slpA B. thuringiensis* activated TLR2 ($P = 0.8857$). Data represents the mean ± SEM for $N \geq 4$ samples).

retained to a significantly greater degree compared with that of WT-infected eyes. The retained responses of A- and B-waves in Δ*slpA*-infected eyes at 12 hours postinfection were approximately 41% and 24%, respectively. Representative waveforms demonstrating the stark differences in A- and B-wave amplitudes of eyes infected with WT or Δ*slpA B. thuringiensis* and uninfected controls at 10 hours postinfection are shown in Figure 3C. Together, these results demonstrated that eyes infected with Δ*slpA B. thuringiensis* retained greater retinal function compared with eyes infected with WT *B. thuringiensis*, suggesting that the presence of SlpA in *B. thuringiensis* influenced the loss of retinal function during experimental endophthalmitis.

Ocular Architecture is Preserved in the Absence of S-layer Protein

A histologic comparison of WT or Δ*slpA B. thuringiensis*-infected C57BL/6J mouse eyes is depicted in Figure 4. At 6, 8, 10, and 12 hours postinfection, eyes were harvested, fixed, sectioned, and stained with H&E. The ocular architecture of uninfected control eyes in both groups was identical, with no signs of inflammation (Fig. 4A). At 6 hours postinfection, the anterior and posterior segments of both WT and Δ*slpA*-infected eyes were similar. At this time point, corneas and posterior segments of infected eyes were not significantly inflamed, retinas were intact, and retinal layers were distinguishable in both groups. At 8 hours postinfection in eyes infected with WT

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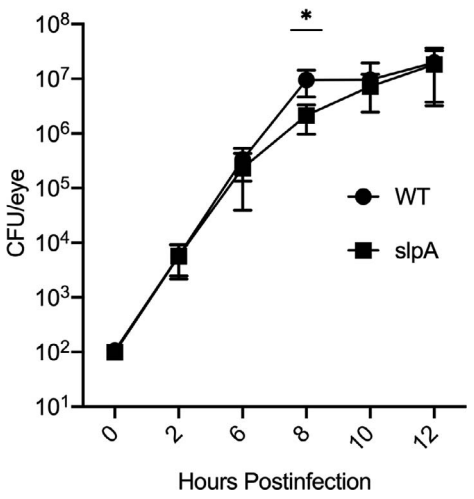

FIGURE 2. *Bacillus* SLP does not influence intraocular growth in endophthalmitis. C57BL/6J mouse eyes were injected with 100 CFU WT *B. thuringiensis* or its isogenic SLP-deficient mutant (Δ*slpA*). At the indicated times postinfection, eyes were harvested and CFU quantified for bacterial intraocular growth. Data represents the mean ± SEM of $\log_{10}$ CFU/eye of $N \geq 4$ eyes per time point for at least two separate experiments. ns: $P > 0.05$ at 0, 2, 6, 10, and 12 hours postinfection. *$P = 0.0087$ at 8 hours postinfection.

B. thuringiensis, significant accumulation of fibrin and infiltrating cells in the posterior segment was observed. Retinas were also partially detached and the anterior chambers were occluded with fibrin. In contrast, ocular structures were well-preserved in Δ*slpA*-infected eyes. These eyes exhibited minimal fibrin deposition and inflammation, intact retinas, and distinguishable retinal layers (Fig. 4A). At 10 hours postinfection, significant fibrin deposition throughout the vitreous and inflammatory cell infiltration were observed in eyes infected with WT *B. thuringiensis*. Corneas in these eyes were highly edematous, and complete retinal detachments and indistinguishable retinal architecture were observed. In stark contrast, retinas were well-preserved and retinal layers were distinguishable in Δ*slpA*-infected eyes at 10 hours postinfection (Fig. 4B). In both infection groups at 10 hours postinfection, bacteria were localized (black arrow) along the posterior capsule, in the midvitreous, and near the inner limiting membrane (ILM) (Fig. 4B). Bacteria were located inside the retinal layers in WT-infected eyes, whereas in Δ*slpA*-infected eyes, no bacteria were found inside the retinal layers and the retinas were intact (Fig. 4B, first column). Bacteria were also located in the anterior chamber and near the iris and ciliary body in eyes infected with either strain (Fig. 4B, third column). At 12 hours postinfection, inflammation pervaded all ocular structures and retinal architecture was completely lost in eyes infected with WT *B. thuringiensis* (Fig. 4A). In eyes infected with Δ*slpA*, retinal layers and architecture remained well-preserved, and these eyes looked similar to that of 6-hour WT-infected eyes (Fig. 4A). These results demonstrated that, in contrast to what was observed in the presence of SlpA, ocular architecture was preserved during *Bacillus* infection, further supporting a contribution of SlpA to the pathogenesis of this disease.

Absence of S-layer Protein Reduces Intraocular Inflammation

We examined the levels of inflammatory cell influx (Fig. 5) in eyes infected with WT or Δ*slpA B. thuringiensis*. The primary infiltrating inflammatory cells in *Bacillus* endophthalmitis are PMN.[1] Infected eyes were harvested at 0, 4, 8, and 12 hours postinfection and PMN influx was estimated by quantifying MPO[85] in eye homogenates by ELISA. MPO concentrations were significantly greater at 4, 8, and 12 hours postinfection in WT-infected eyes compared with that of Δ*slpA*-infected eyes. These results demonstrated that an absence of SlpA in these infections resulted in reduced MPO enzyme levels, indicating a subdued PMN response in these eyes. These results suggest a role for SlpA in the recruitment of PMN into the posterior segment of the eye during infection. Overall, this result corroborates our findings of less retinal function loss and less ocular pathology in eyes infected with SlpA-deficient *B. thuringiensis,* strongly suggesting that SlpA is important to the virulence of this organism during endophthalmitis.

S-layer Protein Activates the NF-κB Pathway in Retinal Cells

Transcription factor NF-κB is a crucial component in human inflammation and disease, and is often considered a target for potential therapeutics.[86] Under normal physiological conditions, inactive NF-κB is present in the cytoplasm as a complex with members of IκB inhibitor family of proteins.[87] When cells receive any multitude of extracellular signals, NF-κB becomes active, enters into the nucleus, and binds to DNA, activating inflammatory gene expression.[86,88] During *Bacillus* endophthalmitis, bacteria have been observed in close proximity to the ILM, as well as within retinal layers when retinas are detached.[2] Because the end feet of retinal Muller cells lie at the ILM/vitreous interface, bacteria in their proximity can adhere to or interact with these surfaces, increasing the possibility of receptor-agonist interactions. Activation of the NF-κB signaling pathway is a sign of receptor-agonist interactions. To determine whether SlpA could activate canonic NF-κB inflammatory pathways, we measured NF-κB DNA binding activity in human retinal Muller cells treated with SlpA from WT *B. thuringiensis*. Figure 6 depicts the percent of NF-κB-DNA binding relative to the untreated control. The activation of NF-κB upon an environmental stimulus is a very rapid process, and we observed a 46% increase in NF-κB-DNA binding within 15 minutes. Maximum DNA binding was achieved at 45 minutes. At 30 and 45 minutes, 84.7% and 96.35% DNA binding were observed, respectively. At 1 and 2 hours in the SLP-treated group, DNA binding was approximately 85%. At all time points, NF-κB-DNA binding was significantly different compared with the untreated control. This result suggests that SLP of *B. thuringiensis* is a potent stimulator of the NF-κB signaling pathway in human Muller cells and may contribute to the regulation of inflammatory modulator release and inflammation.

S-layer Protein Induces the Expression of Inflammatory Modulators From Retinal Cells

Host/pathogen interactions in infections typically involve the activation of the NF-κB pathway, leading to the production of inflammatory mediators to regulate inflammation.[89,90] This is a downstream effect of a receptor/agonist interaction. It has been reported that SLPs of *Lactobacillus helveticus* induce inflammatory mediator production in human and mouse macrophages.[91] Cytokine expression was also observed when mouse bone marrow–derived immature DCs were exposed to SLP from *C. difficile*.[92,93] We therefore analyzed whether SlpA from *B. thuringiensis* induced the expression of inflammatory mediators from human retinal Muller cells. The expression of proinflammatory cytokines IL-6 and TNFα in SlpA-treated Muller cells was significantly greater than that of the extract control (Fig. 7). The expression of proinflammatory chemokines (CCL2 and CXCL-1) was also significantly greater

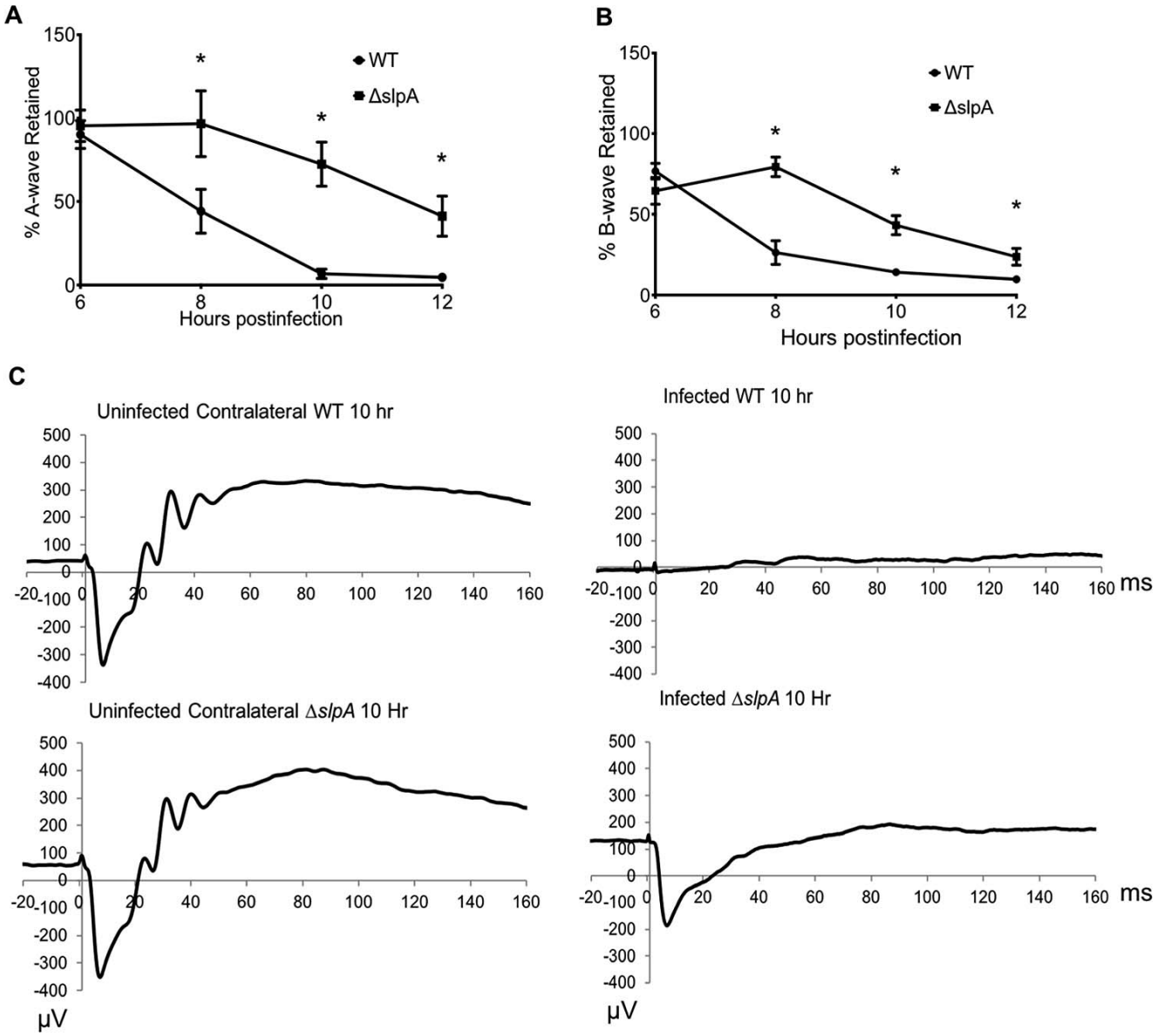

Figure 3. Absence of SLP impacts retinal function loss in *Bacillus* endophthalmitis. C57BL/6J mouse eyes were injected with 100 CFU WT or ΔslpA *B. thuringiensis* and retinal function was assessed by ERG. (**A**) Retained A-wave function was significantly greater in eyes infected with Δ*slpA B. thuringiensis* at 8, 10, and 12 hours postinfection (*$P < 0.04$). (**B**) B-wave function was also significantly greater in eyes infected with Δ*slpA B. thuringiensis* at 8, 10, and 12 hours postinfection (*$P < 0.04$). (**C**) Representative waveforms from eyes infected with WT or Δ*slpA B. thuringiensis* at 10 hours postinfection. In these mice, one eye was infected and the contralateral eye served as the uninfected control. Values represent the mean ± SEM of percentage amplitude retained per time point for at least two separate experiments. Data are representative of $N \geq 8$ eyes per time point.

compared with that of the extract control (Fig. 7). This finding demonstrates that *B. thuringiensis* SlpA induces the expression of inflammatory mediators from human retinal Muller cells, which is an outcome of NF-κB signaling pathway activation.

Discussion

As one of the most immune privileged sites of the body, the eye contains innate defense systems designed to confront and eliminate invading microorganisms without the development of an overwhelming inflammatory response that might disrupt the clarity of visual axis.[94,95] When introduced into the eye, *Bacillus* compromises these defenses, resulting in an explosive inflammatory response. Intraocular inflammation in endophthalmitis caused by members of the *Bacillus cereus sensu lato* group goes hand in hand with severe and irreversible retinal damage. The evolution of endophthalmitis typically depends on the species of the infecting pathogen.[2,31,96] Compared with other pathogens associated with endophthalmitis, intraocular infection with *Bacillus* can reach severe levels quite rapidly. Within 12 to 48 hours, up to two-thirds of *Bacillus*-infected eyes have been reported to have lost useful vision, often leading to enucleation of the eye.[4–10,97,98] The *B. cereus* envelope induced robust intraocular inflammation in a rabbit model of experimental endophthalmitis,[2] presumably due to retinal innate immune recognition of pathogen associated molecular patterns on the organism's surface.[33,34,99,100] Effective therapeutic options for endophthalmitis depend on identifying bacterial and host targets that can be used to modify disease outcomes and prevent vision loss. Therefore,

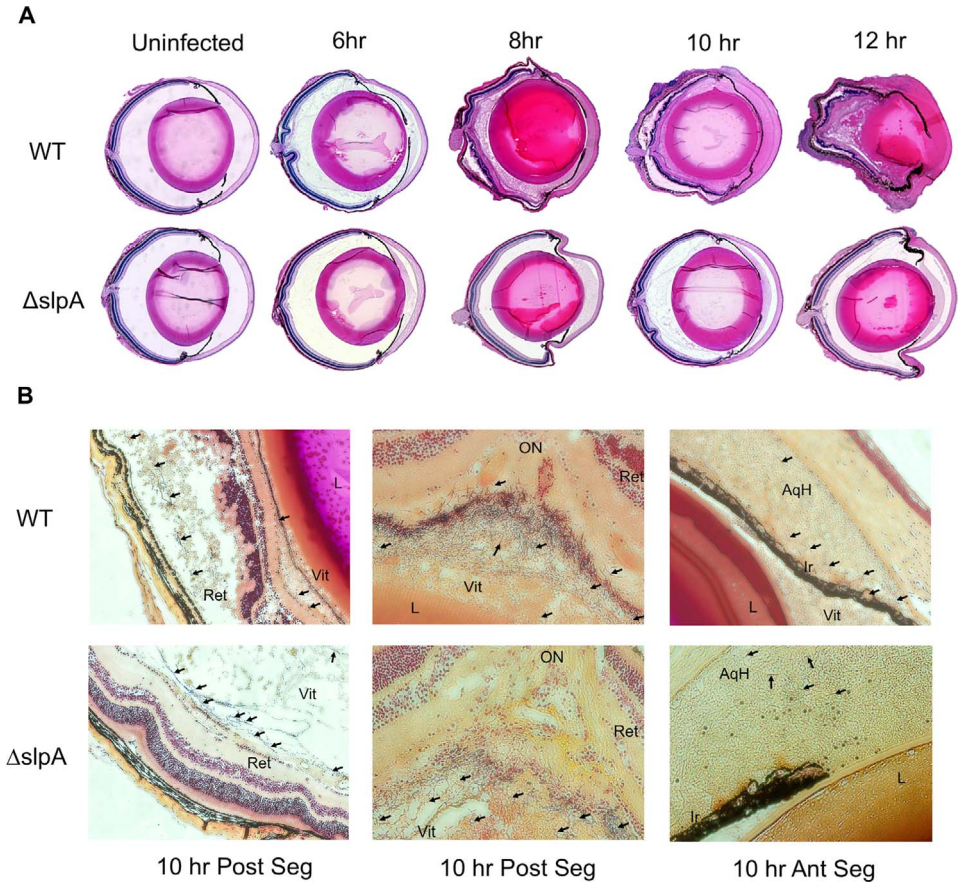

Figure 4. Absence of SLP preserves ocular architecture in *Bacillus* endophthalmitis. (**A**) C57BL/6J mouse eyes were infected with 100 CFU of WT or Δ*slpA B. thuringiensis*. Infected eyes were harvested at 6, 8, 10, and 12 hours postinfection and processed for H&E and Gram staining. Magnification, ×10. (**B**) Bacteria were observed in the midvitreous of eyes infected with each strain at 10 hours postinfection. At this time point, WT and Δ*slpA* mutant were each observed in the aqueous humor of the anterior segment (Ant Seg), as well as in the midvitreous and near the retina of the posterior segment (Post Seg). Sections are representative of three eyes per time point. WT and Δ*slpA B. thuringiensis* are denoted by *black arrows*. Magnification, ×200. Ret, retina; Vit, vitreous; AqH, aqueous humor; Ir, iris; L, lens.

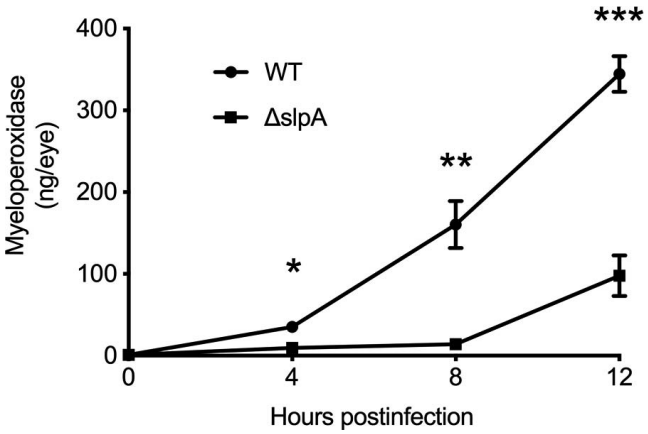

Figure 5. Absence of SLP blunts the intraocular infiltration of PMN during *Bacillus* endophthalmitis. C57BL/6J mouse eyes were injected with 100 CFU WT or Δ*slpA B. thuringiensis*. At 4, 8, and 12 hours postinfection, infected eyes were harvested and infiltration of PMN was assessed by quantifying MPO in whole eyes by sandwich ELISA. MPO levels were significantly greater in eyes infected with WT strains at 4, 8, and 10 hours postinfection compared with the eyes infected with Δ*slpA B. thuringiensis*. Values represent the mean ± SEM of MPO (ng/eye) of $N \geq 4$ per time point for at least two separate experiments. $^{*}P = 0.0095$; $^{**}P = 0.0022$; $^{***}P = 0.0043$.

identifying unique bacterial components that trigger the innate immune response in *Bacillus* endophthalmitis is crucial for understanding the interplay of the host and microbes in this disease.

The outer surfaces of some Gram-positive and -negative bacteria and *Archaea* have a proteinaceous coat known as an SL, formed by the self-assembly of monomeric proteins into a repeatedly spaced, two-dimensional array. As a protective coat, SL contributes not only to bacterial shape, cell wall integrity, and survival, but also to pathogenicity through several mechanisms.[53,56] The function of SL in *Bacillus* endophthalmitis has never been studied. We previously used *B. thuringiensis* as a surrogate pathogen for *B. cereus* endophthalmitis and reported that both pathogens are capable of infecting human, rabbit, and mouse eyes.[23,24,27,79] Here, studying SLP of *B. thuringiensis* will shed light on the role of this protein in *Bacillus* endophthalmitis.

If present, SLPs can constitute up to 15% of the total protein of a bacterial cell during exponential growth.[53,55,56] Using TEM, we confirmed the presence or absence of SL in WT or Δ*slpA B. thuringiensis*, respectively. It has been reported that in artificial growth media, SLP-deficient bacteria grew faster than the WT strain.[43,101] Here, the absence of SlpA did not result in changes in in vitro growth, as WT and Δ*slpA B. thuringiensis* grew at similar concentrations in BHI media. Both strains had similar hemolytic activities on red blood cells and cytotoxic effects on human retinal Muller cells. The SLP of

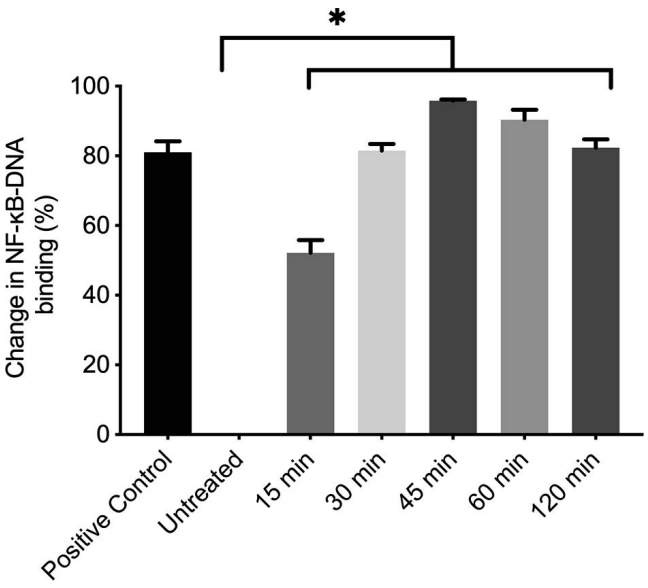

FIGURE 6. *Bacillus* SLP induces NF-κB-DNA binding in human retinal Muller cells. NF-κB-DNA binding in human retinal Muller cells (MIO-M1) treated with 10 μg/mL SlpA for 15, 30, 45, 60, and 120 minutes was quantified by ELISA. The percent of change in NF-κB-DNA binding for each time point relative to untreated control is presented. Values represent the mean ± SEM of change in NF-κB-DNA binding for at least three separate experiments. $^*P = 0.0286$.

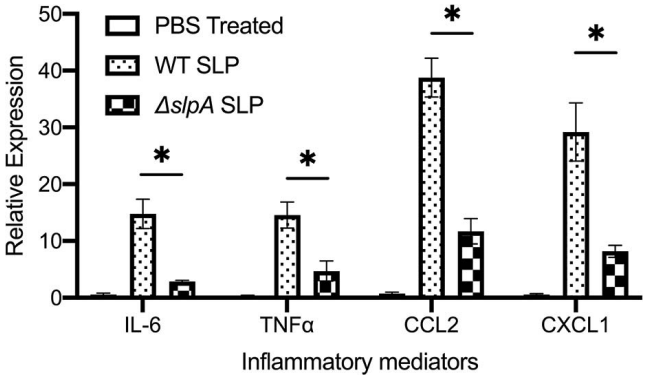

FIGURE 7. *Bacillus* SLP induces the expression of inflammatory mediators in human retinal Muller cells. Expression of proinflammatory cytokines (IL-6, TNFα) and chemokines (CCL2, CXCL-1) from human retinal Muller cells (MIO-M1) was quantified following treatment with 10 μg/mL SlpA or PBS for 10 hours. The "SlpA" fraction from Δ*slpA B. thuringiensis* was used as an extract control. Figures are representative of at least four independent experiments. Relative expression of these genes was determined by RT-PCR using ΔC_T method using GAPDH as a housekeeping gene. Data are represented as the mean ± SEM of gene expression relative to *GAPDH* expression. $^*P = 0.0286$.

Synechococcus, a marine cyanobacteria, was reported to contribute to the motility of this pathogen.[102] However, we found that WT and Δ*slpA B. thuringiensis* were similarly motile. Moreover, we observed that during infection, both WT and Δ*slpA* penetrated the posterior capsule and entered the anterior segment, further supporting the similar motility of these strains. Overall, the absence of SlpA in *B. thuringiensis* resulted in no obvious phenotypic or biochemical differences.

Pathogenic *Bacillus* species and many other pathogenic bacteria have SLPs. SLPs are important virulence factors and may be required for an infection to occur,[43] but the contributions of SLP to bacterial pathogenesis are poorly understood. To study this in the context of experimental endophthalmitis, we compared infections with WT and Δ*slpA B. thuringiensis* in mouse eyes. Intraocular growth of WT and Δ*slpA B. thuringiensis* was not statistically different at most time points, but at 8 hours, less bacilli in Δ*slpA*-infected eyes was observed. We reported a similar observation in experimental endophthalmitis initiated by pili-deficient *B. cereus*.[48] One of the major functions of SLP is to protect the bacteria from phagocytosis and serum killing by binding with complement factor C3b.[56,103] PMN are the primary infiltrating cells during *B. cereus* endophthalmitis[1] and are phagocytic in nature. We reported that *B. cereus* are readily ingested by neutrophils from C57BL/6J mice in vitro.[34] Whether *B. cereus* SLP has any role in protecting *Bacillus* from complement and neutrophil-mediated killing during experimental endophthalmitis is an open question.

The key event in the visual cycle is the phototransduction cascade that occurs within the retina. Therefore, any damage to the retina may cause significant and permanent vision loss or blindness. This is unfortunately a common occurrence in *Bacillus* endophthalmitis. Because the production of bacterial toxins, enzymes, and the inflammatory response in the eye during *Bacillus* endophthalmitis collectively contributes to retinal damage,[23,27,104] we compared the retinal function in the eyes infected with WT or Δ*slpA B. thuringiensis*. Our results demonstrated that an absence of SlpA resulted in significant retention of retinal function, likely due to the preservation of retinal structure in these eyes (Figs. 3, 4). Eyes infected with the WT strain had significant retinal damage and detachments, likely contributing to significant retinal function loss in these eyes. Because WT and SlpA-deficient *B. thuringiensis* had similar cytotoxicity phenotypes, it is unlikely that the stark contrast in retinal damage and function loss were due to differences in toxin production by these strains.

We reported the importance of envelope components of *Bacillus* to intraocular inflammation.[2] Flagella decorate *B. cereus* and *B. thuringiensis* and are essential for motility and migration throughout the eye during endophthalmitis,[23] but are not involved in TLR5-mediated inflammation.[47] The 49-kDa band observed in WT and Δ*slpA* (Fig. 1E) suggests that flagellin may have been present in the Δ*slpA* fractions. However, the presence of flagellin in these preparations was likely negligible based on less severe infections caused by Δ*slpA* (Figs. 3–5), the muted cytokine/chemokine response after exposure to the control Δ*slpA* fraction (Fig. 7), and our report that high, nonphysiological concentrations of flagellin are needed to induce intraocular inflammation.[47]

To date, studies on SLP have not addressed the recruitment of inflammatory cells in any infection model. Here, we demonstrated that a SlpA deficiency blunted the inflammatory response to *Bacillus* infection. Δ*slpA*-infected eyes had significantly less inflammatory influx compared with WT-infected eyes (Figs. 4, 5). The greater retained retinal function and reduced inflammatory influx in Δ*slpA*-infected groups were similar to what we previously observed in immune-deficient (TLR2$^{-/-}$,[33] TLR4$^{-/-}$,[34] MyD88$^{-/-}$,[34] TRIF$^{-/-}$,[34] and CXCL-1$^{-/-}$[74]) mice, suggesting a potential link between SLPs and these innate immune systems. Together, these results suggest that in the absence of *B. thuringiensis* SlpA, a muted inflammatory response led to preserved retinal function.

During infection, *B. cereus* and *B. thuringiensis* migrate from the initial infection site to the midvitreous and localizes in close proximity near the retinal ILM.[2] Here, both WT and Δ*slpA* were present adjacent to the ILM, suggesting a potential interaction between migrating organisms and the retinal cells constituting the ILM. We also observed that SLPs of *B. thuringiensis* induced NF-κB-DNA binding in human retinal

Muller cells and stimulated the production of proinflammatory mediators (IL-6, TNFα, CCL2, and CXCL-1). All of these mediators have been reported to be present in mouse[1,33,34,74,75,83] and human eyes[105] with endophthalmitis. These findings imply that SLPs are immune modulators that may interact with innate immune pathways, and may do so in the eye via Muller cells during endophthalmitis.

Our present findings have demonstrated, for the first time, that the absence of a single virulence factor, the SLP, resulted in a significantly better clinical outcome for experimental *Bacillus* endophthalmitis. Because current anti-inflammatory therapeutics used this disease (i.e., corticosteroids) are relatively ineffective in altering rapidly evolving inflammation and subsequent vision loss,[15,16,31,32] it is important to identify the bacterial components that interact with innate immune pathways to trigger this robust inflammation. Due to its rapidly blinding course, *Bacillus* endophthalmitis requires prompt and accurate treatment, which should be based largely on an understanding of the bacterial and host factors, which contribute to disease pathogenesis. Further understanding of the mechanisms by which SLPs contributes to the pathogenesis of *Bacillus* endophthalmitis could be instrumental in the development of new therapeutic regimens to block inflammation and prevent vision loss during this blinding infection.

Acknowledgments

The authors thank Mark K. Coggeshall, Tarea Burgett, and Alanson W. Girton (Oklahoma Medical Research Foundation) for stimulating discussions on peptidoglycan purification, Feng Li and Mark Dittmar (OUHSC Live Animal Imaging Core, Dean A. McGee Eye Institute, Oklahoma City, OK, USA) for invaluable technical assistance, and Excalibur Pathology (Moore, OK, USA) and the OUHSC Cellular Imaging Core (Dean A. McGee Eye Institute, Oklahoma City, OK, USA) for histology expertise. The human Muller cell line (MIO-M1) was a kind gift from Astrid Limb (Institute of Ophthalmology, Moorfields Eye Hospital, London).

Supported by National Institutes of Health grants R01EY028810 and R01EY024140 (to MCC). Our research is also supported in part by National Institutes of Health grants R01EY025947 and R21EY028066 (to MCC), National Eye Institutes Vision Core Grant P30EY027125 (to MCC), a Presbyterian Health Foundation Research Support Grant Award (to MCC), a Presbyterian Health Foundation Equipment Grant (to Robert E. Anderson, OUHSC), and an unrestricted grant to the Dean A. McGee Eye Institute from Research to Prevent Blindness.

Disclosure: **M.H. Mursalin**, None; **P.S. Coburn**, None; **E. Livingston**, None; **F.C. Miller**, None; **R. Astley**, None; **A. Fouet**, None; **M.C. Callegan**, None

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