Aging, But Not Sex and Genetic Diversity, Impacts the Pathobiology of Bacterial Endophthalmitis

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Purpose. Age, sex, and genetics are important biological variables in determining an individual's susceptibility or response to infectious agents; however, their role has not been evaluated in intraocular infections. In this study, we comprehensively examined the impact of these host biological factors in the pathogenesis of experimental bacterial endophthalmitis.

Methods. Endophthalmitis was induced by intravitreal injection of bacteria (*Staphylococcus aureus*) in the eyes of male and female C57BL/6 mice of different ages: group I (young, 6–8 weeks), group II (mid-age, 18–20 weeks), and group III (old, 1 year). Highly heterogeneous outbred J:DO mice were used for genetic diversity analysis. Eyes were subjected to clinical examination, retinal function testing using electroretinography (ERG), histopathological analysis (hematoxylin and eosin staining), and bacterial burden estimation. The levels of inflammatory mediators were measured using qPCR and ELISA, and the infiltration of neutrophils was determined by flow cytometry.

Results. Both inbred C57BL/6 and diversity outbred (J:DO) mice were equally susceptible to *S. aureus* endophthalmitis, as evidenced by a time-dependent increase in clinical scores, bacterial burden, intraocular inflammation, and retinal tissue damage, in addition to decreased retinal function. However, no significant differences were observed in disease severity and innate responses in male versus female mice. Older mice (group III) exhibited higher clinical scores coinciding with increased bacterial proliferation and intraocular inflammation, resulting in enhanced disease severity. Moreover, bone-marrow-derived macrophages from old mice exhibited reduced phagocytic activity but increased inflammatory response toward *S. aureus* challenge.

Conclusions. Age, but not sex, is an important biological variable in bacterial endophthalmitis. Identification of pathways underlying altered innate immunity and impaired bacterial clearance in aging eyes could provide new insights into the pathobiology of intraocular infections in elderly patients.

Keywords: *S. aureus*, endophthalmitis, aging, male, female, diversity outbred, J:DO, inflammation, retina, ERG

Postoperative intraocular infections, such as bacterial endophthalmitis, although rare, are of great concern to ophthalmologists because of their ability to cause rapid vision loss if not diagnosed and treated promptly.[1,2] Historically, elevated incidences of endophthalmitis are primarily associated with cataract surgery; this correlation might be due to the larger volume of cataract surgeries performed worldwide or to the unavailability of newer technologies for minimally invasive procedures in the developing world.[3–6] The prevalence of ocular diseases increases considerably with age, and management of these diseases typically requires invasive procedures such as intravitreal injections.[7,8] Therefore, all ocular surgical procedures increase the chances for developing bacterial endophthalmitis in patients.[9,10] Several epidemiological studies have shown that the severity of bacterial endophthalmitis depends on the virulence of infecting pathogens.[11–13] In addition, host factors such as genetic composition, immunologic status, sex, and age are also key determinants of susceptibility and response to invading pathogens.[14] However, to our knowledge, no clinical or experimental studies have systematically evaluated the impact of these biological variables on the incidence or severity of bacterial endophthalmitis. This prompted us to carry out

the current study using experimental models of bacterial endophthalmitis.

Many studies have suggested that the manifestation and morbidity of infectious and inflammatory diseases differ between the sexes and among different ages. In general, males are more susceptible to diverse bacterial and viral infections in comparison to females, and this sexual dimorphism is apparent throughout all stages of life.[15–17] These variations in the susceptibility and severity of infections between males and females have been attributed to genetic, biological, and behavioral differences, which include various factors such as exposure to certain pathogens, sex hormones, and differences in innate immune response.[16,18,19] In females, the X chromosome harbors several genes implicated in the immune response, such as genes encoding Toll-like receptors, cytokine receptors, transcription factors, and proteins that control T and B cell function, ultimately providing females with a stronger innate and adaptive immune response than their male counterparts.[19–21] In both males and females, sex hormones modulate the immune response by regulating nuclear factor kappa B and mitogen-activated protein kinase pathways.[16,22–24] In addition to immune system modulation, sex hormones have been shown to influence bacterial growth, metabolism, and virulence factors.[22,25–27]

Aging also influences the immune system, and advancing age increases susceptibility toward infectious diseases. Aging has a direct effect on ocular complications and is linked to various age-related diseases such as age-related macular degeneration (AMD), diabetic retinopathy, and glaucoma. Age-related changes in the cornea and ocular surface tissue have been shown to affect vision.[28] Nevertheless, how aging influences ocular bacterial infection remains elusive. In older age, diminished phagocytic activity by dendritic cells, impaired antigen presentation, and activation of the adaptive immune system can lead to increased susceptibility to infections.[29–31] Similarly, due to thymic involution, the naïve T-cell repertoires are manipulated, and differentiated effector T cells can produce more proinflammatory cytokines, which, in combination with activated innate immune cells, contribute to a systemic proinflammatory milieu in the elderly population.[32,33] The decline in sex hormones due to aging has also been linked to decreased immune cell numbers and function, thus further increasing susceptibility to infections.[33,29]

Despite acknowledging the impact of biological variables such as sex and age on various diseases, there is a lack of studies on ocular infections, and they are almost nonexistent with regard to bacterial endophthalmitis. Thus, in this study, we assessed the host biological variables that resulted in key differences in the pathobiology of bacterial endophthalmitis and demonstrated an important role of aging, but not sex or genetic diversity.

Materials and Methods

Ethics Statement

All mice experiments were performed in strict adherence to the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee (IACUC) of Wayne State University approved the protocol (ID IACUC-19-03-1012). Mice were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed under anesthesia using a ketamine/xylazine cocktail or isoflurane.

Mice

Inbred C57BL/6 mice of both sexes and of various ages (young, 6–8 weeks; mid-aged, 18–20 weeks; old, 1 year) and the outbred J:DO (Generation 35) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in a pathogen-free Division of Laboratory Animal Resources facility at the Kresge Eye Institute. Mice were maintained in a 12-hour light/12-hour dark cycle at 22°C and fed LabDiet rodent chow (Labdiet PicoLab, St. Louis, MO, USA) and water ad libitum.

Induction of Bacterial Endophthalmitis

Endophthalmitis was induced in mice as described previously.[34,35] Briefly, mice were anesthetized and intravitreally injected with *Staphylococcus* (*S.*) *aureus* strain RN6390 (5000 CFU/eye in 2-µL volume of PBS) using a 32-gauge needle under an ophthalmoscope. Eyes injected with PBS served as controls. Eye exams were performed using an ophthalmoscope fitted with a camera for clinical scoring and imaging. Ocular health was graded by assigning clinical scores ranging from 0 to 4 (least to severe flare/haze/opacity), as per the previously described criteria.[36,37]

Bacterial Burden Estimation

Following infection, at indicated time points, mouse eyes were enucleated, and whole-eye lysates were prepared in PBS by homogenization using stainless steel beads in a tissue lyser (Qiagen, Valencia, CA, USA). The tissue homogenate was serially diluted and plated on Tryptic Soy Agar and incubated at 37°C. The following day, colonies were counted, and results were expressed as the mean number of CFU/eye ± SD.

RNA Extraction and Quantitative Real-Time PCR

Retinal tissues from control and bacterial infected eyes were removed and pooled (two retinas per sample) in TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) for RNA extraction. Total RNA was reversed transcribed using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) per the manufacturer's instruction. Quantitative real-time PCR (qPCR) was performed using inflammatory cytokine and chemokine gene-specific primers on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data were analyzed using the comparative $\Delta\Delta C_T$ method. The gene expression in the test samples was normalized to endogenous β-actin controls.

Enzyme-Linked Immunosorbent Assay

The levels of intraocular inflammatory cytokines and chemokines were determined using commercially available mouse ELISA kits. Whole-eye lysates were prepared by homogenization using a tissue lyser as described above, and an equal amount (20 µg) of total protein was used for the desired cytokine and chemokine ELISA

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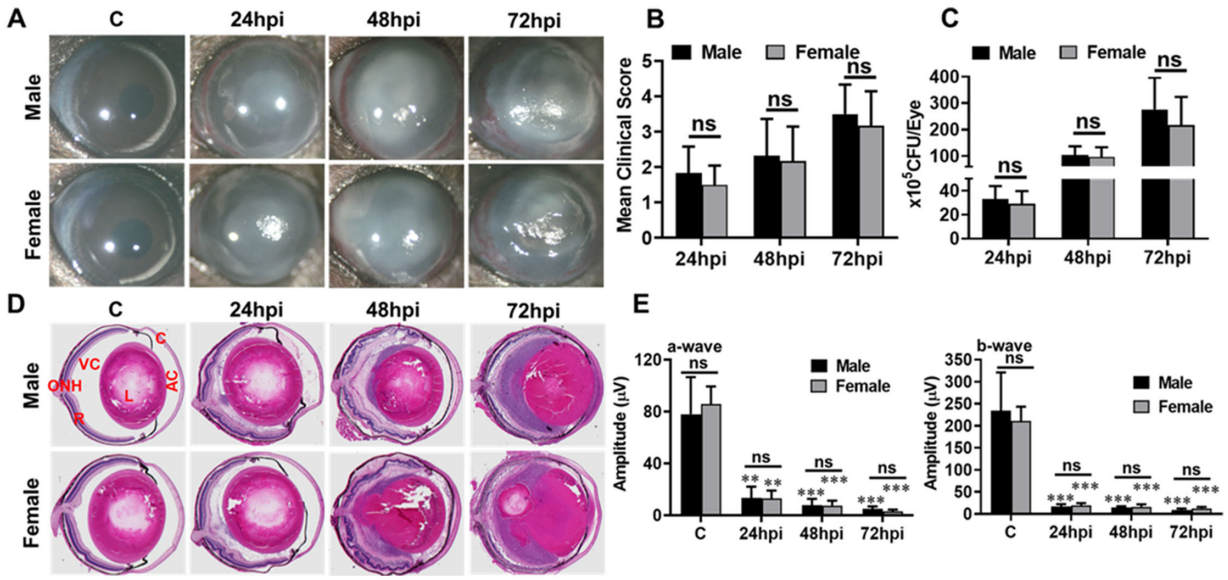

Figure 1. Both male and female C57BL/6 mice are equally susceptible to *S. aureus* endophthalmitis. Eyes of C57BL/6 male and female mice (6–8 weeks of age, n = 10–12 each sex) were infected with *S. aureus* (SA), strain RN6390 (5000 CFU/eye), by intravitreal injection. Eyes with PBS injection were used as control (C). (**A**) Representative micrograph shows that SA induced corneal haze/opacity in the infected eyes. (**B**) Disease progression was graded on a five-point clinical scoring system (score of 0 indicating a healthy eye and scores of 1–4 indicating increasing severity). (**C**) At indicated time points, the whole-eye lysates were used to estimate the intraocular bacterial burden by serial dilution and the plate count method (represented as CFU/eye). (**D**) Enucleated eyes were fixed and stained with H&E at the indicated time points. (**E**) Scotopic ERG was performed at the indicated time points. The bar graph represents the a- and b-wave amplitudes retained at the indicated time points. Statistical analysis was performed using Student's *t*-test (male vs. female; **B**, **C**) or two-way ANOVA with Tukey's multiple comparison test (control vs. SA and male vs. female; **E**). $^{**}P < 0.005$; $^{***}P < 0.0005$. C, cornea; AC, anterior chamber; L, lens; VC, vitreous chamber; R, retina; ONH, optic nerve head; ns, not significant.

assays as per the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The data are presented as mean cytokine or chemokine concentrations (pg/mg of eye lysates) ± SD.

Histology

Following euthanasia, eyes were enucleated and fixed in 4% formalin for histopathological examination. The embedding, sectioning, and hematoxylin and eosin (H&E) staining were performed by Excalibur Pathology, Inc. (Oklahoma City, OK, USA), and the PathScan Enabler IV (Meyer Instruments, Inc., Houston, TX, USA) was used to scan the H&E stained slides.

Electroretinography Analysis

Scotopic electroretinography (ERG) was performed to assess retinal visual function. ERG was performed in control and *S. aureus*-infected mouse eyes using the Celeris ERG system (Diagnosys LLC, Lowell, MA, USA) per the manufacturer's recommendations.

Flow Cytometry

The infiltration of polymorphonuclear leukocytes (PMNs) was determined using flow cytometry as described in our prior studies.[35,38,39] Briefly, at desired time points, retinas (two retinas were pooled) were harvested from enucleated mouse eyes, and single-cell suspensions were prepared by digesting with Accumax (MilliporeSigma, Burlington, MA, USA) for 10 minutes at 37°C followed by trituration through a 23-gauge needle and syringe. Tissue debris was removed by filtering through a 40-µm cell strainer (BD Biosciences, San Jose, CA, USA) followed by rinsing the cells with 0.5% BSA in PBS and incubating with BD Fc Block (BD Biosciences) for 30 minutes. After blocking, the cells were rinsed with 0.5% BSA and incubated with CD45–PECy5 and Ly6G–FITC antibodies (BD Biosciences) in the dark for 30 minutes. After two washes, cells were acquired using a BD Accuri C6 Flow Cytometer (BD Biosciences) and analyzed using Accuri C6 software.

Bone-Marrow-Derived Macrophage Isolation

Bone-marrow-derived macrophages (BMDMs) from old and young mice were isolated as described previously.[40,41] Briefly, bone marrow was flushed from femurs and tibias using a 25-gauge needle and syringe filled with RPMI media containing 10% FBS and 0.2-mM EDTA. Cells were pelleted by centrifugation at 400*g* for 5 minutes at 4°C. Following red blood cell (RBC) lysis, cell pellets were washed with RPMI media by centrifugation at 400*g*. Cells were suspended and seeded in RPMI media supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 ng/mL macrophage colony-stimulating factor for macrophage differentiation at 37°C in 5% CO_2. Six days post-differentiation, ~1 × 10^6 BMDMs/mL were seeded in six-well tissue-culture dishes for in vitro experiments.

Phagocytosis Assay

Bacterial phagocytosis assays were performed using BMDMs from young and old mice as described previously.[42] Briefly,

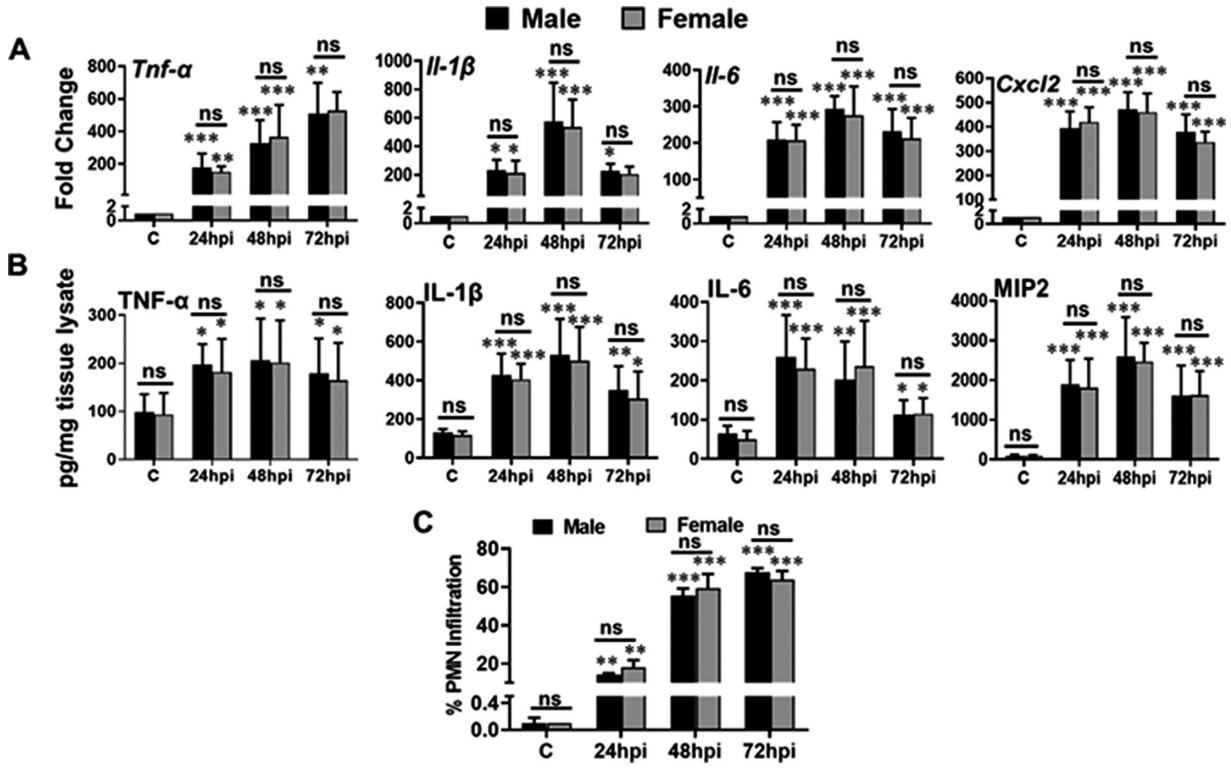

Figure 2. The inflammatory response in C57BL/6 mice is not influenced by sex bias. C57BL/6 male and female mice (6–8 weeks of age, $n = 10$ each sex) were infected with *S. aureus* (SA), strain RN6390 (5000 CFU/eye), by intravitreal injection. Eyes with PBS injection were used as control. (**A**) At the designated time points, the retinas were harvested and subjected to qPCR for the indicated cytokine or chemokine. (**B**) The whole-eye lysates were subjected to ELISA to measure the protein levels of the indicated cytokines or chemokines. (**C**) At the indicated time points, neural retinas were harvested, and single-cell suspensions were stained with anti-CD45–PECy5 and anti-Ly6G–FITC antibodies to estimate PMN infiltration. The bar graph represents the percent retinal PMN infiltration. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparison test (control vs. SA and male vs. female). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

BMDMs (10^6 cells/well) were grown in 60-mm Petri dishes in the Dulbecco's modified Eagle's medium (DMEM) medium. The cells were infected with *S. aureus* (multiplicity of infection [MOI], 10:1) in each Petri dish and incubated for 2 hours. Following incubation, the cells were washed and treated with gentamicin (200 µg/mL) for 2 hours to kill all extracellular and/or adherent bacteria. Two hours after the gentamicin was added, the cells were washed with DMEM and incubated in fresh DMEM containing gentamicin (200 µg/mL) for 1 hour and 24 hours. For the enumeration of phagocytized bacteria, following incubation, the cells were washed three times with PBS and lysed with 0.01% Triton X-100. The lysed cells were scraped and washed by centrifugation at 5000*g* for 5 minutes. The pellets were resuspended in 1 mL of sterile PBS and serially diluted, plated, and counted for viable bacterial counts.

Statistical Analysis

Statistical analyses were performed using Prism 8.1.2 (Graph Pad, San Diego, CA, USA). All data are expressed as mean ± SD unless indicated otherwise. Statistical significance was determined using either two-way ANOVA or unpaired *t*-tests, as indicated in the figure legends. $P < 0.05$ was considered statistically significant.

Results

Sex Differences Do Not Influence the Pathobiology of Bacterial Endophthalmitis

To investigate the role of sex as a biological variable, eyes of both male and female C57BL/6 mice (6 to 8 weeks old) were challenged with *S. aureus* to induce endophthalmitis as described earlier.[34,35,43] As expected, *S. aureus* infection resulted in non-resolving endophthalmitis indicated by a time-dependent increase in corneal haze, anterior chamber opacity, and hypopyon, but no significant difference was observed in clinical scores of male versus female mice (Figs. 1A, 1B). The bacterial burden estimation showed a time-dependent increase from 24 hours post-infection (hpi) to 72 hpi, but their levels were similar in both sexes at any given time point (Fig. 1C). The histopathological analysis (Fig. 1D) revealed that, in comparison with uninfected control eyes (Fig. 1C), *S. aureus*-infected eyes had profound retinal tissue damage that coincided with reduced retinal function as assessed by ERG (Fig. 1E, Supplementary Fig. S1A). Like other parameters, there was no marked difference in histological and ERG response in infected eyes of male versus female mice.

As sex has been shown to influence immune response,[19–21,44] we next sought to assess intraocular inflammation by measuring the expression of inflammatory

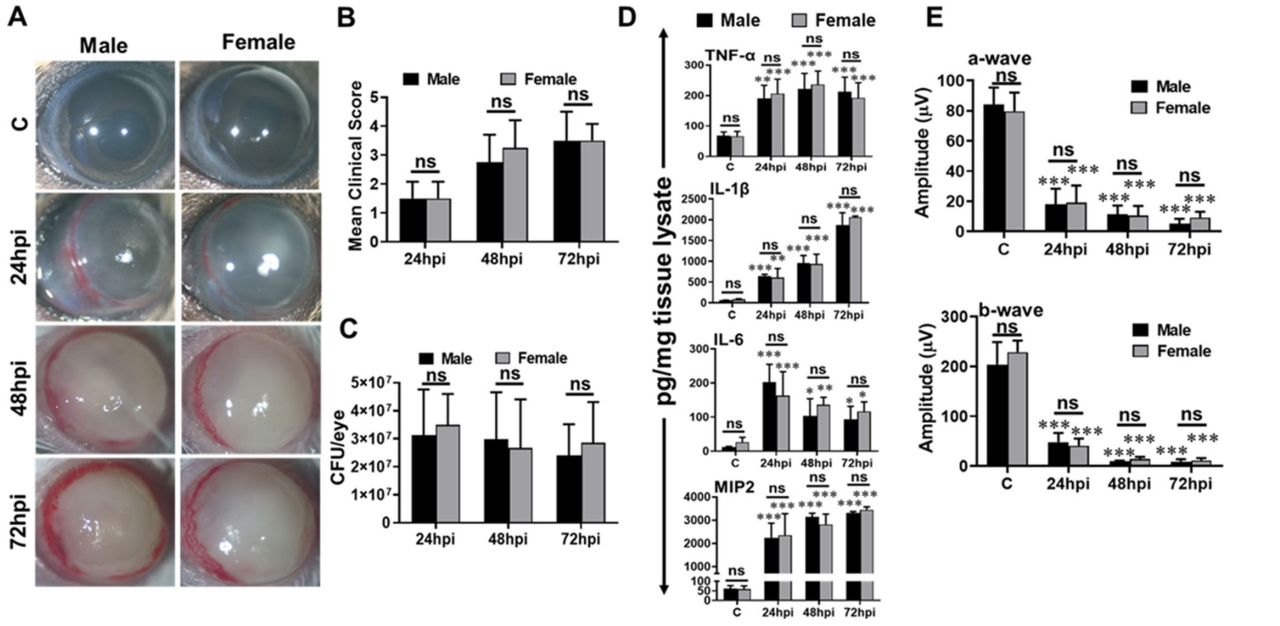

Figure 3. Genetic diversity does not influence a sex bias to *S. aureus* endophthalmitis. Eyes of J:DO male and female mice (6–8 weeks of age, n = 8–10 each sex) were infected with *S. aureus* (SA), strain RN6390 (5000 CFU/eye), by intravitreal injection. Eyes with PBS injection were used as control. (**A**) Representative micrograph shows that SA induced corneal haze/opacity in the infected eyes. (**B**) Disease progression was graded on a five-point clinical scoring system. (**C**) At the indicated time points, the whole-eye lysates were used to estimate the intraocular bacterial burden by serial dilution and the plate count method (represented as CFU/eye). (**D**) The whole-eye lysates were subjected to ELISA to measure the protein levels of the indicated cytokines or chemokines. (**E**) At the designated time points, scotopic ERG was performed, and the data are presented as amplitudes of the a- and b-waves. Statistical analysis was performed using Student's *t*-test (male vs. female; **B**, **C**) or two-way ANOVA with Tukey's multiple comparison test (control vs. SA and male vs. female; **D**, **E**). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

mediators in infected eyes. To this end, our data showed that *S. aureus* induced the expression of the inflammatory cytokines TNF-α, IL-1β, and IL-6, as well as the chemokine CXCL2/MIP2, at both the transcript (Fig. 2A) and protein (Fig. 2B) levels. However, the comparative analysis revealed no sex-based differences in the induction of innate inflammatory responses. Moreover, the infiltration of neutrophils (PMNs) to the retina was also not influenced by sex, as evidenced by similar levels of PMNs in both male and female mice (Fig. 2C, Supplementary Fig. S1B). Collectively, these results indicate that inbred C57BL/6 mice do not exhibit sex-specific differences during bacterial endophthalmitis.

Genetic Diversity Is a Dispensable Biological Variable in Bacterial Endophthalmitis

Because we did not observe any sex-based differences in disease pathology in inbred C57BL/6 mice, we decided to test whether genetic diversity plays any role as a biological variable in sex bias. To investigate this, we used diversity outbred (J:DO) mice, a novel outbred strain designed to preserve founder genomes and prevent allelic loss. The J:DO founders were derived from eight inbred strains, including traditional laboratory strains such as C57BL/6 and A/J, as well as three wild-derived mice strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ) through collaborative cross.[45–47] As these mice have been used to account for genetic diversity in bacterial and viral infections,[46,47] *S. aureus* endophthalmitis was induced in both male and female J:DO mice (6–8 weeks old). Our time-course study revealed that, like C57BL/6 mice, J:DO mice developed severe endophthalmitis resulting in a time-dependent increase in corneal haze, anterior chamber opacity, and hypopyon (Fig. 3A). Sex-based comparative analyses revealed no significant difference in clinical scores (Fig. 3B), bacterial burden (Fig. 3C), production of inflammatory mediators (Fig. 3D), ERG response (Fig. 3E, Supplementary Fig. S2) in *S. aureus*-infected eyes of male and female J:DO mice. These findings indicate that genetic diversity does not influence sex bias in the disease susceptibility toward bacterial endophthalmitis.

Aging Increased the Severity of Bacterial Endophthalmitis Without Sex Bias

Because bacterial endophthalmitis is commonly associated with ocular surgeries that are performed mainly in the elderly, we next sought to determine age as a biological variable. As our first experiment using young mice (6–8 weeks old) showed no sex differences, we postulated that there might be differences in older mice and decided to use mid-age C57BL/6 mice (18–20 weeks old) to induce *S. aureus* endophthalmitis. However, no significant difference was observed in mid-age male versus female mice in terms of the gross clinical exam (Fig. 4A), disease severity (Fig. 4B), bacterial burden (Fig. 4C), histology (Fig. 4D), or retinal function (Fig. 4E, Supplementary Fig. S3). Likewise, no sex-based differences were evident in the production of inflammatory mediators (Supplementary Fig. S4A) or retinal PMN infiltration (Supplementary Figs. S4B, S4C). Interestingly, when age-based comparisons were made, the mid-age

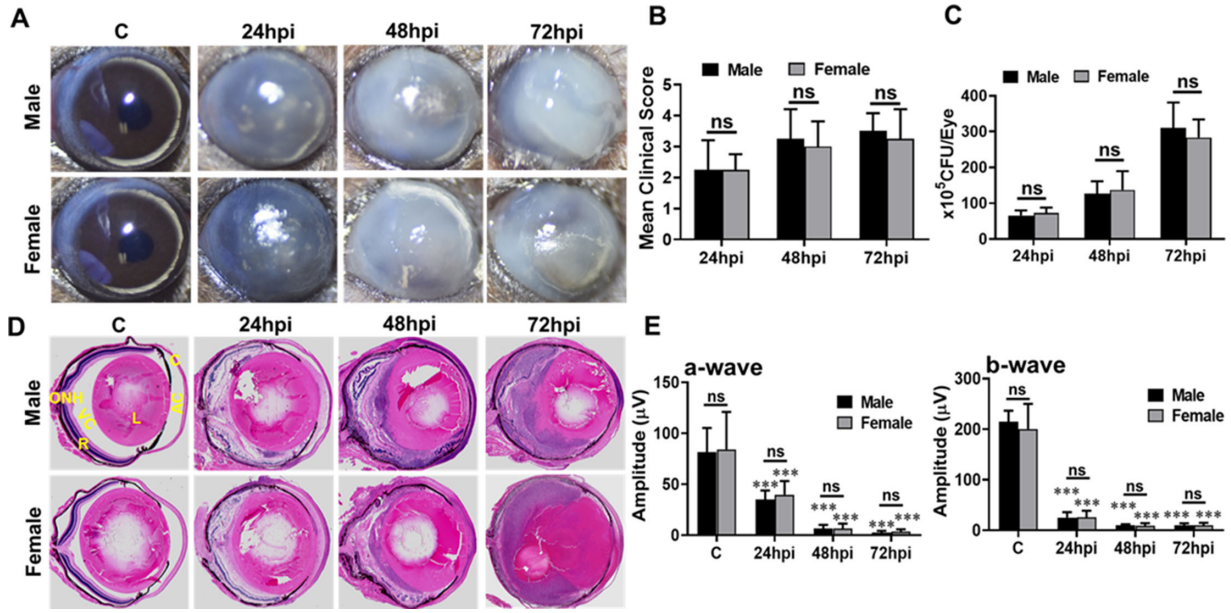

Figure 4. Aging does not change the sex bias in *S. aureus* endophthalmitis. Mid-age (18–20 weeks of age) C57BL/6 male and female mice eyes (n = 8–10 each sex) were infected with *S. aureus* (SA), strain RN6390 (5000 CFU/eye), by intravitreal injection. Eyes with PBS injection were used as control. (**A**) Representative micrograph shows that SA induced corneal haze/opacity in the infected eyes. (**B**) Disease progression was graded on a five-point clinical scoring system and represented as a mean clinical score. (**C**) At the indicated time points, the whole-eye lysates were used to estimate the intraocular bacterial burden by serial dilution and the plate count method (represented as CFU/eye). (**D**) At the indicated time points, eyes were enucleated, fixed, and stained with H&E. (**E**) Scotopic ERG was performed at the indicated time points, and the data are presented as amplitudes of the a- and b-waves. Statistical analysis was performed using Student's t-test (male vs. female; **B**, **C**) or two-way ANOVA with Tukey's multiple comparison test (control vs. SA and male vs. female; **E**). $^{***}P < 0.0005$.

Table. Disease Parameters in Young Versus Mid-Age Mice

Disease Parameters	Time Points Post-Infection (hpi)	Young Mice (6–8 wk), Mean ± SD		Mid-Age Mice (18–20 wk), Mean ± SD	
		Male	Female	Male	Female
Clinical score	24	1.8 ± 0.75	1.5 ± 0.54	2.25 ± 0.96	2.25 ± 0.5
	48	2.33 ± 1.03	2.16 ± 0.98	3.25 ± 0.96	3 ± 0.82
	72	3.5 ± 0.83	3.1 ± 0.98	3.5 ± 0.58	3.25 ± 0.96
Bacterial burden (CFU)	24	$33.21 \pm 10.64 \times 10^5$	$29.08 \pm 10.62 \times 10^5$	$66.55 \pm 14.4 \times 10^5$	$72.42 \pm 15.54 \times 10^5$
	48	$103.03 \pm 33.89 \times 10^5$	$97.21 \pm 35.79 \times 10^5$	$126.36 \pm 34.79 \times 10^5$	$137.21 \pm 52.44 \times 10^5$
	72	$276.54 \pm 120.44 \times 10^5$	$216.71 \pm 106.84 \times 10^5$	$309.87 \pm 71.3 \times 10^5$	$283.38 \pm 50.45 \times 10^5$

mice exhibited increased disease severity as compared to the younger mice, evident by increased clinical scores and the intraocular bacterial burden (Table). Similarly, the mid-age mice exhibited higher levels of IL-1β in comparison to the younger mice.

We observed an increasing trend in the clinical score and bacterial burden and some inflammatory mediators in young versus mid-age animals, we decided to investigate the disease pathology in older mice. For this, 1-year-old C57BL/6 mice were compared with young mice (6–8 weeks of age), which are routinely used in our laboratory. Because we did not find sex-based differences among our young, mid-age, and diversity outbred mice, we decided to use only female mice for this comparison. As shown in Figure 5A, *S. aureus* induced endophthalmitis in both young and old mice; however, the disease was more severe in the old mice as compared to the young mice. The young mice had less corneal haze, anterior chamber opacity, and hypopyon at 24 and 48 hpi as compared to the old mice; however, disease severity increased with time for both old and young mice (Fig. 5A). The mean clinical scores also showed significant differences in the young versus old mice at early time points (24–48 hpi): young mice, 1.5 ± 0.54 at 24 hours and 2.16 ± 0.98 at 48 hours; old mice, 3 ± 0.63 at 24 hours and 3.6 ± 0.5 at 48 hours. In contrast, clinical scores were comparable at 72 hpi: young mice, 3.16 ± 0.98; old mice, 3.83 ± 0.40 (Fig. 5B). Coinciding with clinical scores, the bacterial burden was significantly higher in old mice at all time points (Fig. 5C). A similar trend was observed for the inflammatory cytokines and chemokines (IL-1β, IL-6, and CXCL2/MIP2) at both the transcript (Fig. 5D) and protein (Fig. 5E) levels, with significantly higher levels in old versus young mice. Finally, retinal function analysis revealed that, in the old mice, the b-wave amplitude was significantly reduced

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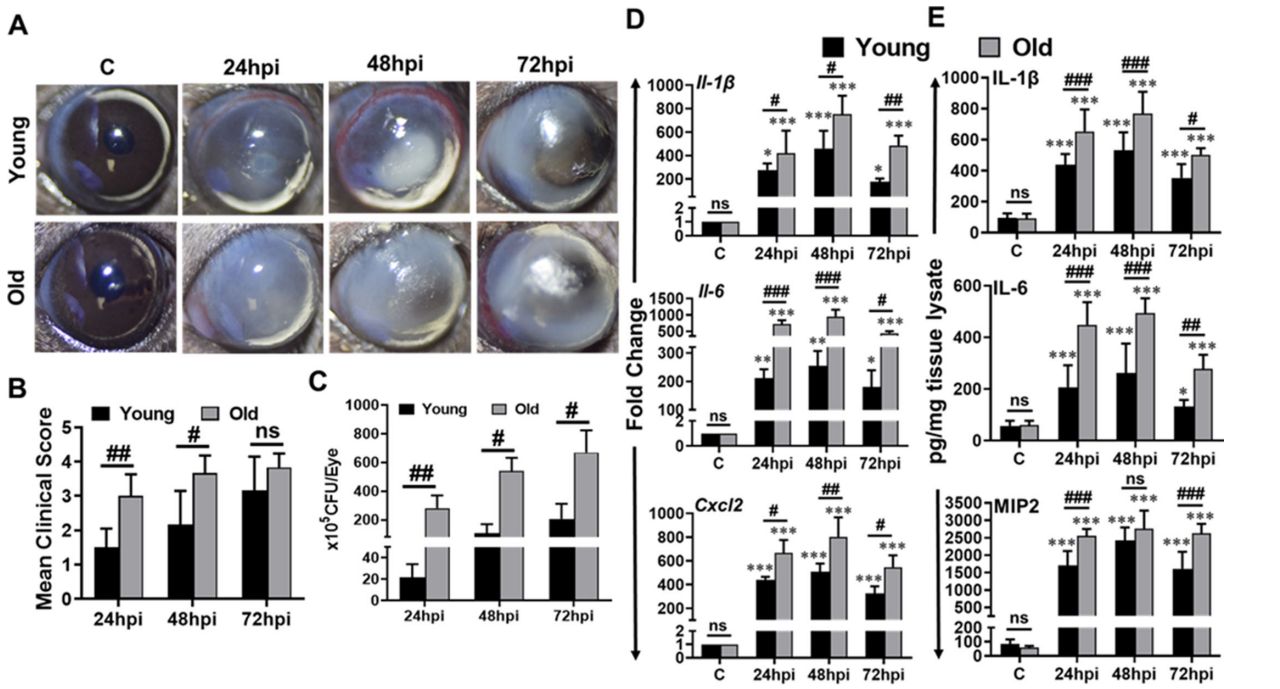

Figure 5. Aging increases disease severity in *S. aureus* endophthalmitis. Old (1 year of age, $n = 10$) and young (6–8 weeks of age, $n = 10$) C57BL/6 female mice eyes were infected with *S. aureus* (SA), strain RN6390 (5000 CFU/eye), by intravitreal injection. Eyes with PBS injection were used as control. (**A**) Representative micrograph shows that SA induced corneal haze/opacity in the infected eyes. (**B**) Disease progression was graded on a five-point clinical scoring system and represented as a mean clinical score. (**C**) At the indicated time points, the whole-eye lysates were serially diluted and plated for intraocular bacterial burden estimation (represented as CFU/eye). (**D**) At the designated time points, the retinas were harvested and subjected to qPCR to measure the mRNA transcripts of the indicated cytokines or chemokines. (**E**) The whole-eye lysates were subjected to ELISA to measure the protein levels of the indicated cytokines or chemokines. Statistical analysis was performed using Student's *t*-test (male vs. female; **B**, **C**) or two-way ANOVA with Tukey's multiple comparison test (control vs. SA and male vs. female; **D**, **E**). $^{\#,*}P < 0.05$; $^{\#\#,**}P < 0.005$; $^{\#\#\#,***}P < 0.0005$.

at 24 and 48 hpi as compared to the young mice; whereas, at 72 hpi, both old and young mice had a comparable b-wave response (Fig. 6). In contrast, no significant difference was observed in the a-wave amplitudes. Our data also showed slightly decreased b-wave amplitudes in the uninfected old control mice as compared to the young mice, indicating that aging affects the b-wave amplitude.

Aging Impaired Bacterial Phagocytic Activity and Increased Inflammatory Response of Macrophages

Aging has been shown to modulate immune cell functions. In bacterial endophthalmitis, infiltrating myeloid cells such as macrophages and neutrophils play a key role in the pathogenesis. To further investigate the effect of aging on myeloid cells in bacterial infection, we used BMDMs from old (1 year) and young (6–8 weeks of age) mice and challenged with *S. aureus*. We discovered that BMDMs from the old mice had significantly elevated levels of cytokines and chemokines following bacterial infection as compared to their younger counterparts at transcript (Fig. 7A) and protein (Fig. 7B) levels. Moreover, BMDMs from the old mice exhibited reduced phagocytic uptake and intracellular bacterial killing as compared to the young mice (Fig. 7C). These results indicate that aging affects myeloid cell function. Collectively, these findings suggest that aging is a crucial biological variable influencing the pathobiology of bacterial endophthalmitis.

Discussion

Both clinical and experimental studies have shown that the severity of endophthalmitis depends on virulence factors of the infectious agent.[48,49] Thus, the visual outcome in bacterial endophthalmitis caused by coagulase-negative staphylococci such as *Staphylococcus epidermidis*[50] is better as compared to that for *S. aureus*[34,43] or *Bacillus cereus*.[11] However, host–pathogen interactions are more complex where an avirulent or a commensal microbe can cause disease in immunocompromised individuals, and an immunocompetent host could be protected even from virulent pathogens.[51] On the one hand, the host immune response to pathogens is a common denominator that can translate into different disease outcomes; on the other, immune responses could vary depending on the sex and age of the host. Indeed, several funding agencies, including the National Institutes of Health, and journals are now mandating sex-/gender-based analyses to improve rigor and reproducibility in biomedical research.[52] To our knowledge, there are no studies available that have systematically evaluated the effect of these biological variables in bacterial endophthalmitis.

In this study, using a mouse model of *S. aureus* endophthalmitis, we have shown that aging is an important determinant of disease outcome, but sex and genetic diversity

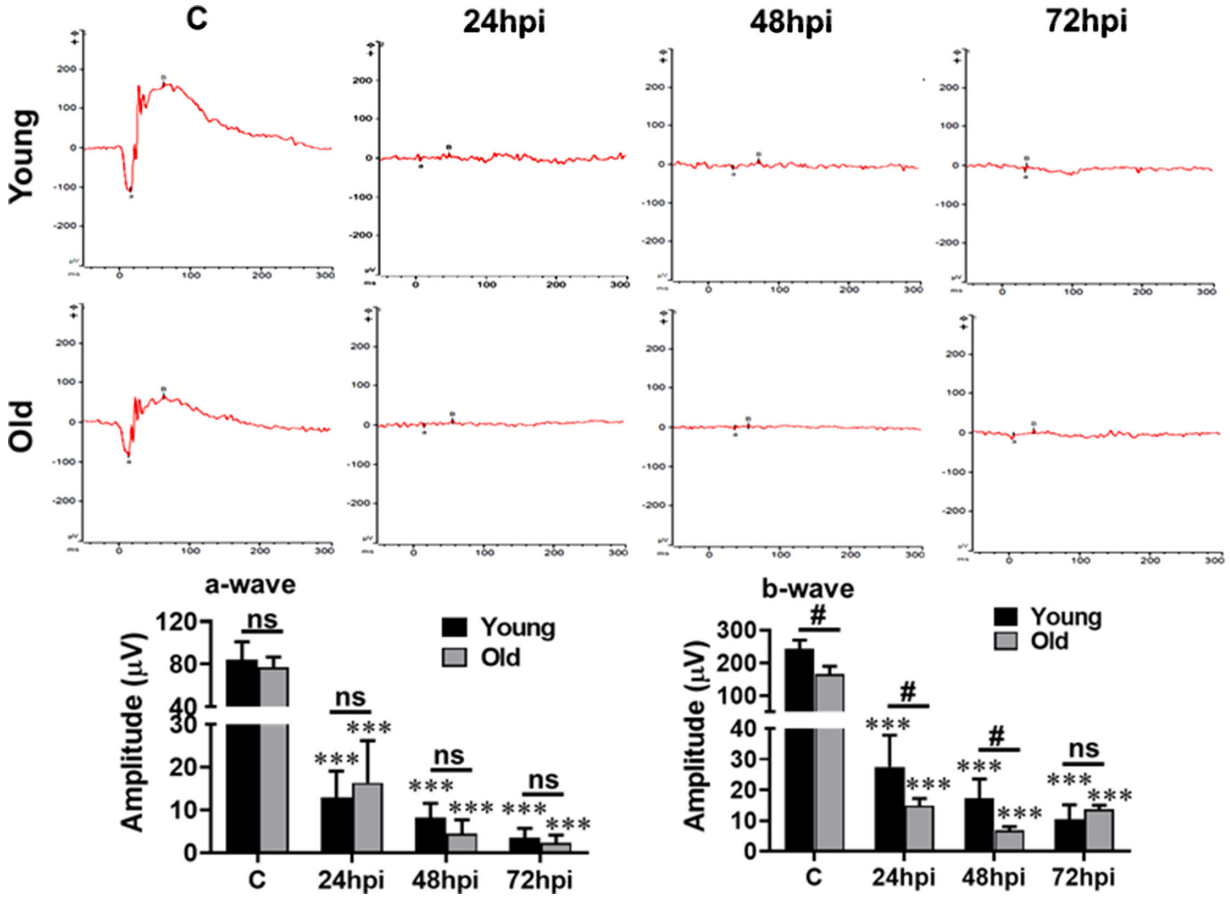

Figure 6. Aging modulates retinal function in bacterial endophthalmitis. Old (1 year of age) and young (6–8 weeks of age) C57BL/6 female mice eyes ($n = 10$) were infected with *S. aureus* (SA), strain RN6390 (5000 CFU/eye), by intravitreal injection. Eyes with PBS injection were used as control. Scotopic ERG was performed at the indicated time points. The bar graph represents the a- and b-wave amplitudes retained at the respective time points. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparison test (control vs. SA and young vs. old). $^{\#}P < 0.05$; $^{***}P < 0.0005$.

do not affect the severity of bacterial endophthalmitis. Our data showed that, regardless of age, both male and female mice showed similar disease pathology, as evidenced by an equal degree of corneal haze/opacity, clinical score, and bacterial burden in their eyes following *S. aureus* infection. However, in *S. aureus* skin infection models, female mice were found to be more resistant in comparison to males with reduced dermal necrosis and bacterial burden.[53] In another study, male mice have been shown to be more prone to *S. epidermidis*-induced hypoxic–ischemic brain injury in neonates as compared to females.[54] Similarly, in pneumococcal sepsis and pneumonia, male mice exhibited increased susceptibilities.[55] In contrast, for herpes simplex virus 1 (HSV-1) keratitis, no sex-based differences were observed in viral burden and tissue damage.[56] HSV-2 infection, however, has shown a sex bias in human studies where women had a higher prevalence of infection than men.[57] We postulate that the immune-privileged status of the eye may account for no sex bias in bacterial endophthalmitis, as sex steroid hormones might not influence the immune response in this disease.

Previous studies from our lab, as well as by other investigators, have shown that the severity of endophthalmitis is strongly correlated with intraocular inflammation, characterized by the infiltration of neutrophils and production of inflammatory cytokines.[35,37–39,49,58] In general, due to differences in sex hormones, males present Th1 dominance by producing TNF-α, IL-1β, and IL-6 cytokines, whereas females activate a Th2 response and produce high levels of interleukins (IL-4, IL-5, and IL-10) in response to infection.[21,59–63] Here, our sex-based analysis did not reveal any significant difference in the production of inflammatory mediators and neutrophil infiltration in the infected eyes. Our study corroborated a HSV-1 keratitis study where sex bias did not influence corneal immune cell infiltration and inflammation.[56] Moreover, our histology and retinal function data revealed no significant differences in young and mid-age male versus female mice and support the idea that sex does not play a major role in the disease outcome in bacterial endophthalmitis.

We did not observe any sex-based differences in disease pathology in bacterial endophthalmitis, we considered evaluating the effect of sex biases with genetic diversity as biological variables. Notably, most studies have used an inbred mouse as an experimental model for infectious diseases; however, inbred lines have been shown to exhibit strain-specific characteristics. Indeed, our recent study showed that BALB/C mice are more susceptible to *Candida* endophthalmitis as compared to C57BL/6 mice.[64] Our study, using diversity outbred (J:DO) mice, suggests

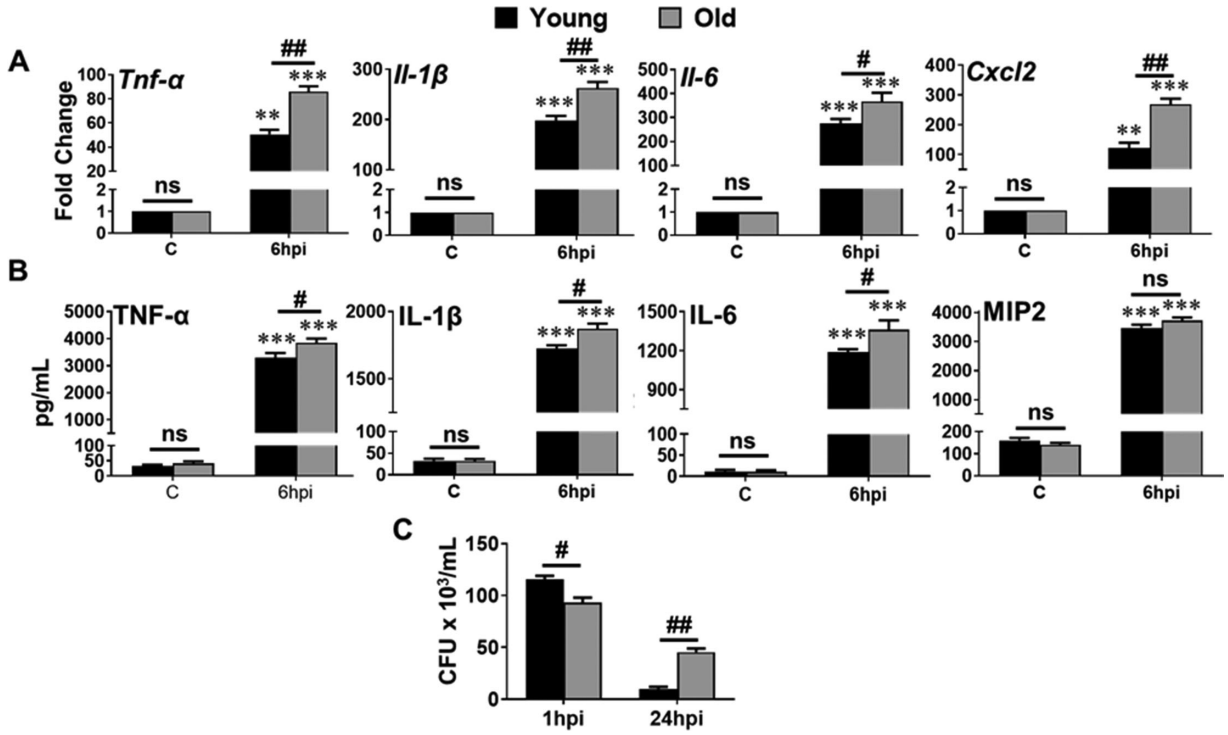

Figure 7. BMDMs from aged mice exhibit increased inflammatory response and impaired antibacterial activity. (**A**) BMDMs from young (6–8 weeks of age) and old (1 year of age) mice were seeded in six-well plates and infected with *S. aureus* (SA) (MOI 10:1) for 6 hours. Uninfected cells were used as control. Following infection, both control and SA-infected cells were subjected to qPCR for the indicated inflammatory mediators. (**B**) Conditioned media were subjected to ELISA to quantify protein levels of the indicated cytokines and chemokines. (**C**) To assess the phagocytic and intracellular killing activity, BMDMs from young and old mice were challenged with SA for 2 hours. After 2 hours of infection, cells were rinsed to remove extracellular bacteria and incubated with fresh medium containing gentamicin (200 µg/mL) up to the indicated time points. At the desired time point, cells were lysed, and the viable bacterial counts were quantitated via serial dilution and plate count. At 1 hour, more CFU were observed in BMDMs from young mice, indicating that the number of internalized bacteria was greater as compared to the old mice, whereas at 24 hours there were more CFU in BMDMs from the old mice compared to their younger counterparts, indicating increased intracellular killing by BMDMs from the younger mice. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparison test (control vs. SA and young vs. old). Data represent mean ± SD from three independent experiments. $^{\#,*}P < 0.05$; $^{\#\#,**}P < 0.005$; $^{\#\#\#,***}P < 0.0005$.

that, like the inbred C57BL/6 strain, J:DO mice developed *S. aureus* endophthalmitis, but no noticeable differences in clinical scores or bacterial burden were observed in male versus female J:DO mice. Interestingly, we observed a slight increase in bacterial burden in J:DO as compared to C57BL/6 mice. In a longitudinal study, J:DO mice were found to be more susceptible to *Mycobacterium tuberculosis*, as they exhibited increased lung bacterial load and pathology and died 80 days post-infection, whereas B6 mice survived until 113 days.[47] Although no sex-based differences were observed in innate responses, *S. aureus*-infected J:DO mouse eyes had slightly elevated levels of IL-1β and CXCL2/MIP2 as compared to C57BL/6 mice, whereas TNF-α and IL-6 levels were similar. In systemic bacterial infections, diversity outbred mice have been shown to evoke a strong chemokine response.[46,65]

Endophthalmitis can occur at any age, whether it is post-traumatic, postoperative, or endogenous, but the majority of postoperative endophthalmitis occurs following cataract surgery, which is performed mainly on the elderly population. Although a few retrospective studies have reported a variable incidence of endophthalmitis in ages ranging from 1 to 81 years,[66–68] studies evaluating the effect of aging in the pathobiology of infectious endophthalmitis are lacking. As elderly people are highly susceptible to microbial infections, including those caused by *S. aureus*,[69–71] we sought to determine the effect of age in bacterial endophthalmitis. Indeed, aging is an underlying cause of several eye diseases such as cataracts, AMD, dry eye diseases, and diabetic retinopathy. Because the majority of endophthalmitis studies, including those from our lab, have used 6- to 8-week-old C57BL/6 mice, we decided to use mid-age mice (18–20 weeks old) to compare the disease pathology. We observed that aging increased levels of inflammatory mediators and other disease parameters in infected eyes of young versus mid-age mice, but there was no sex-based difference, as both male and female mice exhibited similar ocular pathology.

To further validate the effect of aging on the pathogenesis of bacterial endophthalmitis, we decided to compare young mice (6–8 weeks of age) with old mice (1 year old), and we observed marked differences. We found that the old mice had increased ocular pathology compared to the young mice, as evidenced by increased clinical scores and intraocular bacterial burdens. Similarly, the old mice exhibited increased levels of the proinflammatory cytokines IL1-β and IL-6 and the chemokine MIP2 in comparison to the young mice. The effect of aging has been evaluated in some ocular infections. Using a *Pseudomonas keratitis* model, a study has reported increased IFN-γ production in

younger mice, whereas IL-1β levels were elevated in the older mice at 24 hours post-infection.[72] The bacterial burden showed a significant difference only at the 24-hour time point with increased CFU in the older mice. Our in vitro data for cultured BMDMs from the old mice indicate increased production of inflammatory cytokines and reduced bacterial phagocytic activity. Thus, we postulate that impaired or dysregulated innate immune cell function might be responsible for the observed age-based differences in our study. The increased susceptibility of older people to bacterial endophthalmitis or potentially worse disease outcomes could also be in part due to the liquefaction of vitreous which allows increased spread of the bacteria in the vitreous cavity.

Conclusions

To the best of our knowledge, our study is the first to demonstrate that, among the key biological variables (age, sex, and genetic diversity), only age-based differences observed, with older mice exhibiting increased intraocular inflammation and higher bacterial burden. However, further studies are needed to identify biological pathways and mechanisms underlying age differences in the pathobiology of bacterial endophthalmitis. These new insights would enable us to develop specific adjunct therapies (e.g., non-immunosuppressive, antiinflammatory) for older individuals which are currently limited to antibiotics only.

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PKS and AK conceived the idea and designed the experiments. PKS, SS, and REW performed the experiments, analyzed the data, and prepared the figures. PKS and AK wrote the manuscript. RR contributed aged mice, provided intellectual inputs, and critically reviewed the manuscript. AK contributed reagents, materials, analysis tools, and funding for the study. All authors reviewed and approved the final version of the manuscript.

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