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## Glycolytic Inhibitor 2-Deoxyglucose suppresses inflammatory response in Innate Immune Cells and Experimental Staphylococcal endophthalmitis

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### Abstract

Previously, we have shown that *Staphylococcus (S) aureus* induces a glycolytic response in retinal residential (microglia) and infiltrated cells (neutrophils and macrophages) during endophthalmitis. In this study, we sought to investigate the physiological role of glycolysis in bacterial endophthalmitis using a glycolytic inhibitor, 2-deoxy-glucose (2DG). Our data showed that 2DG treatment attenuated the inflammatory responses of mouse bone marrow-derived macrophages (BMDM) and neutrophils (BMDN) when challenged with either live or heat-killed *S. aureus* (HKSA). Among the inflammatory mediators, 2DG caused a significant reduction in levels of cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-6) and chemokines (CXCL1 and CXCL2). Western blot analysis of 2DG treated cells showed downregulation of bacterial-induced MEK/ERK pathways. *In vivo*, intravitreal administration of 2DG both pre- and post- bacterial infection resulted in a significant reduction in intraocular inflammation in C57BL/6 mouse eyes and downregulation of ERK phosphorylation in retinal tissue. Collectively, our study demonstrates that 2DG attenuates inflammatory response in bacterial endophthalmitis and cultured innate immune cells via inhibition of ERK signaling. Thus glycolytic inhibitors in combination with antibiotics could mitigate inflammation-mediated tissue damage in ocular infections.

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#### Authors Contribution

RF, PKS, and SS designed, performed the experiments, analyzed the data, and prepared the figures. PKS and AK wrote the manuscript. AK conceived the idea and provided direction and funding for the project. SG provided intellectual inputs and critically reviewed the manuscript. All authors reviewed and edited the manuscript.

#### Disclosure

The authors have no financial conflicts of interest.

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## Keywords

*S. aureus*; Endophthalmitis; Retina; Inflammation; Glycolysis; 2DG

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## 1. Introduction

Infectious endophthalmitis is a devastating ocular complication caused by a wide range of microbes and can result in visual impairment or blindness if not treated promptly. Gram-positive bacteria, specifically Staphylococci, which typically enters the eye during ocular surgeries (e.g., cataract surgery) and/or trauma, are the leading cause of the majority of endophthalmitis cases. The incidence rate of post-operative endophthalmitis ranges from 0.01% to 0.87% while post-traumatic endophthalmitis occurs in 3–17% of cases following open globe injury (Cailegan et al., 1999; Callegan et al., 2002; Sadaka et al., 2012; Talreja et al., 2015; Miller et al., 2019; Thompson et al., 1995). In addition, intravitreal injections, commonly used for delivery of therapeutic modalities in the eyes, increase the risk of developing endophthalmitis (Sadaka et al., 2012; Thompson et al., 1995). Once, inside the eye, retinal residential cells (glial cells) trigger inflammatory signaling as a host defense mechanism to eradicate invading pathogens by increasing the infiltration of myeloid cells, such as neutrophils and monocytes/macrophages (Gupta et al., 2019; Miller et al., 2019; Talreja et al., 2015). If unchecked, this inflammatory cascade can result in inflammation-mediated ocular tissue damage resulting in vision loss or blindness (Callegan et al., 2007; Miller et al., 2019; Talreja et al., 2015). Current treatment regimens use antibiotics and, if severe, vitrectomies are performed. Even with these medical and surgical treatment options, 1/3 of cases will result in decreased visual acuity (Callegan et al., 2002; Callegan et al., 2007; Sadaka et al., 2012; Singh et al., 2014a; Talreja et al., 2015). In an attempt to identify new treatment strategies, our laboratory's interest has been on discovering therapeutic modalities which take into consideration both eradicating invading pathogens and limiting intraocular inflammation (Kumar et al., 2016; Kumar and Kumar, 2015; Kumar et al., 2013; Singh et al., 2014a).

Using transcriptomic and system biology approaches, our prior studies demonstrate that bacterial and viral infections dysregulate several metabolic pathways in retinal cells (Rajamani et al., 2016; Singh et al., 2018). We previously reported *Staphylococcus aureus* (SA) infection increased glycolysis in both retinal microglia as well as BMDM. Moreover, an AMP-activated protein kinase (AMPK) activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), reduced the glycolytic response and protected the mouse retina from SA induced damage (Kumar et al., 2016). Similarly, AMPK activation was found to restrict Zika virus replication in endothelial cells by inhibiting glycolysis and potentiating innate anti-viral response (Singh et al., 2020). Collectively, these findings from our lab and others highlight the importance of modulating cellular metabolism to improve the outcome of wide range of diseases (Dasgupta et al., 2017; Leung et al., 2012; O'Neill et al., 2016). Furthermore, many immune cells, such as T-cells (Pearce et al., 2009; Shi et al., 2011), NK-cells (Mah et al., 2017) and macrophages (Galvan-Pena and O'Neill, 2014; Kumar et al., 2016), utilize glycolysis during their effector functions by allowing cells to quickly obtain energy and biosynthetic intermediates needed to fight against infection. Because pro-

inflammatory cells utilize glycolysis during their effector phase, this pathways could potentially be targeted to limit host driven inflammatory damage.

The glycolytic inhibitor, 2-deoxy-glucose (2DG), has been best characterized in animal models and widely used to investigate the role of glycolysis in several diseases (Brown, 1962; Sun et al., 2016). In cancer cell lines, 2DG slowed down cell proliferation and apoptosis (Cheong et al., 2011; Dasgupta et al., 2017). Similarly, inhibition of T-cell glycolysis prevented graft rejection (Lee et al., 2015) and reduced excessive IFN- $\gamma$  production in autoreactive CD4+ T cells (Yin et al., 2015). Furthermore, 2DG has been shown to alter macrophage functions such as cytokine production, and phagocytosis (Galvan-Pena and O'Neill, 2014; Michl et al., 1976) and modulation of several infectious diseases (Leung et al., 2012; Varanasi et al., 2017; Wang et al., 2016). However, to our knowledge, the role of glycolysis has not been investigated in ocular bacterial infections.

Since increased inflammation causes retinal tissue damage in bacterial endophthalmitis and infectious/inflammatory conditions have been linked to increased glycolytic activity, we hypothesize that inhibition of glycolysis may exert anti-inflammatory effects. Herein, we investigated the role of a glycolytic inhibitor, 2DG, on innate immune cells and in a mouse model of staphylococcal endophthalmitis.

## 2. Materials and Methods

### 2.1. Bacterial strain and Reagents

*S. aureus* (strain RN6390) was maintained in tryptic soy broth and agar plates (TSB/TSA; Sigma-Aldrich, St. Louis, MO). 2-Deoxy-glucose was purchased from Cayman Chemical (Ann Arbor, MI). For *in vitro* experiments cells were pretreated with 10mM of 2DG for 1 h followed by indicated infection times. For *in vivo* applications, the left eyes of each mouse was injected with 25  $\mu\text{g}/\text{eye}$  (1 $\mu\text{l}$  volume) of 2DG, 14–16 h prior to or 6 h post bacterial infection. The dose of 2DG for intravitreal injection was chosen based on preliminary dose (10–100  $\mu\text{g}/\text{eye}$ ) titration. Contralateral eyes with sterile PBS injection were used as a controls. An ERK inhibitor, U1260, was purchased from InvivoGen (San Diego, CA) and cells were pretreated for 1 h with 10  $\mu\text{M}$  of U1260. Antiphospho-ERK and ERK antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Anti-HSP90 antibody was purchased from Cell Signaling Technology (Beverly, MA). Secondary horseradish peroxidase (HRP)-conjugated anti-mouse or antirabbit IgG antibodies were purchased from Bio-Rad (Hercules, CA).

### 2.2. Mice Infection and Ethics Statement

C57BL/6 (wild type [WT]) was purchased from Jackson Laboratory (Bar Harbor, ME). Both male and female mice, 6 to 8 weeks of age, were used in all the experiments. Mice were housed in a restricted-access Division of Laboratory Animal Resources (DLAR) facility, maintained on a 12 h light 12 h dark cycle at 22°C temperature, and fed on LabDiet rodent chow (PicoLab; LabDiet, St. Louis, MO) and water *ad libitum*. Mice were treated in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures

were approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University. Endophthalmitis was induced in the mice by intravitreal injection of *S. aureus* RN6390 (5000 CFU/eye). Eyes injected with sterile PBS served as controls. Following infection at endpoints, eyes were enucleated and subjected to bacterial growth determination, inflammatory cytokine/chemokine, and immunoblot assays.

### 2.3 Electroretinography

Scotopic electroretinography (ERG) was used to determine retinal function in *S. aureus* infected and 2DG treated mouse eyes using Celeris ERG system (Diagnosis LLC, Lowell, MA), as described previously (Guest et al., 2018; Singh et. al. 2014a)

### 2.4 Isolation of bone marrow-derived macrophages (BMDM) and neutrophils (BMDN)

Mice BMDM and BMDN were isolated as described previously (Kumar et al., 2016; Swamydas and Lionakis, 2013). Briefly, femurs and tibias were isolated from euthanized mice. Bone marrow was flushed 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 x g for 5 min at 4°C. RBCs were lysed by adding a hypotonic solution of 0.2% NaCl for 20 sec, followed by the addition of 1.6% NaCl and centrifugation. Following RBC lysis, cell pellets were washed with RPMI media by centrifugation at 400 x g. Cells were resuspended and seeded in RPMI media supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 ng/ml M-CSF for macrophage differentiation at 37°C in 5% CO<sub>2</sub>. Six days post differentiation ~1×10<sup>6</sup> BMDM/mL were seeded in 6 well tissue-culture plates for *in-vitro* experiments.

For BMDN extraction, bone marrow cell pellets were resuspended in 1 ml PBS and overlaid over a histopaque gradient of 1119 and 1077 (Sigma-Aldrich, St. Louis, MO), followed by centrifugation at 600 x g at 25°C for 30 min without centrifuge break. The layer between histopaques 1119 and 1077 was collected and washed two times with RPMI media by centrifugation at 400 x g for 7 min and BMDN cells were cultured as described for macrophages. For *in vitro* infections, cells were challenged with *S. aureus* RN6390 at Multiplicity of Infection (MOI) 10:1 for indicated time points. For heat-killed bacteria, similar MOI of *S. aureus* is boiled for 5 min and used for *in vitro* experiments.

### 2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of the inflammatory cytokines and chemokines in the *in vitro* culture supernatant and mice eye lysates (15–20 µg total protein) were determined using commercially available ELISA kits. ELISA was performed for inflammatory cytokines: TNF-α, IL-1β, IL6, and chemokines: CXCL2 (MIP2), and CXCL1 (KC) as per manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). Data were presented as mean ± standard deviation (SD) pg/ml for *in vitro* conditioned media, and mean ± SD pg/mg of eye lysates.

### 2.6. RNA Extraction and qRT-PCR Analysis

Total RNA was then extracted from cells using TRIzol reagent, as per the manufacturer's instruction (Invitrogen, Carlsbad, CA). cDNA was synthesized using 1 µg of total RNA using a Maxima first-strand cDNA synthesis kit, as per the manufacturer's instructions

(ThermoFisher Scientific, Rockford, IL). Quantitative RT-PCR was conducted in a StepOnePlus instrument (Applied Biosystems, Grand Island, NY, USA) using cDNA for pro-inflammatory cytokines and chemokines, *Tnf- $\alpha$* , *Il-1 $\beta$* , *Il6*, *Cxcl11*, and *Cxcl2* genes. TaqMan primers were purchased from Integrated DNA Technologies (Coralville, IA). The quantification of gene expression was determined by the comparative CT method. Expression levels in the test samples were normalized to those of endogenous reference  $\beta$ -*actin* levels. Data are mean  $\pm$  SD fold change.

## 2.7. Immunoblotting

Following incubation, cells were washed with ice-cold PBS and lysed using RIPA buffer containing protease inhibitor cocktail. Samples were sonicated briefly, followed by centrifugation at 15,000 x g for 20 min. Total protein was quantified using a Micro BCA protein assay kit (ThermoFisher, Rockford, IL). Total protein samples (30  $\mu$ g) were resolved on a 12% SDS-PAGE in Tris-glycine-SDS buffer and electro-blotted onto a nitrocellulose membrane (BioRad, Hercules, CA). Blots were blocked for 1 h at room temperature with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skimmed milk. The membrane was probed with primary antibodies diluted in 3% BSA in TBST overnight at 4°C. After three washes with TBST, the membrane was incubated with anti-mice/rabbit HRP conjugated secondary antibody diluted in TBST with 5% milk at room temperature for 2 h. Protein bands were developed with SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher Scientific, Rockford, IL), and visualized using iBright FL1500 Imaging Systems (ThermoFisher Scientific, Rockford, IL).

## 2.8. Statistics

All data have been expressed as means  $\pm$  SD unless indicated otherwise. All statistical analysis was performed using GraphPad Prism V8 (GraphPad Software, La Jolla, CA). One-way ANOVA was used for group comparisons followed by Tukey's Multiple Comparison post-hoc test. A *P*-value of <0.05 was considered statistically significant. All experiments were performed at least three times unless indicated otherwise.

## 3. Results

### 3.1. 2DG attenuates SA-induced inflammatory response in macrophages and neutrophils

Previously, we have shown that *S. aureus* infection induced a glycolytic response in retinal microglia and mouse retina (Kumar et al., 2016). To ascertain the role of glycolysis in bacterial-induced inflammation, we assessed the effect of a glycolytic inhibitor, 2DG, *in vitro* using BMDM (Fig. 1) and BMDN (Fig. 2). In response to live SA challenge, BMDM exhibited induced expression of inflammatory cytokines *Tnf- $\alpha$* , *Il-1 $\beta$* , *Il-6*, and chemokines *Cxcl11*, *Cxcl2* mRNA transcripts as compared to control (Fig. 1A). 2DG treatment attenuated the SA-induced expression of all of these cytokines/chemokines { *Tnf- $\alpha$* , *Il-1 $\beta$* , *Il-6*, *Cxcl11*, and *Cxcl2* } transcripts. Concomitant with mRNA expression, levels of these pro-inflammatory mediators, at protein levels were also increased by live SA infection and reduced by 2DG treatment except CXCL2 protein levels (Fig. 1B). BMDM challenged with HKSA also resulted in induced expression of various inflammatory mediators, however, their levels were relatively low compared to live SA infection. In HKSA challenged BMDM,

2DG treatment attenuated mRNA expression of *Tnf- $\alpha$* , and *Il-1 $\beta$*  but not *Il-6*, *Cxcl1* and *Cxcl2*. A similar trend was observed at protein levels of these inflammatory mediators with 2DG reducing levels of TNF- $\alpha$  and IL-6 but not IL-1 $\beta$ , CXCL1, and CXCL2.

To determine whether the anti-inflammatory effects of 2DG are cell-specific, bone marrow-derived neutrophils (BMDN) were challenged with live or HKSA. Likewise, in BMDN, live SA challenge significantly induced the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, CXCL1, and CXCL2 both at transcripts (Fig. 2A) as well as protein levels (Fig. 2B) and 2DG treatment attenuated this response. In BMDN challenged with HKSA, 2DG treatment only reduced the expression of cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 and had no significant effect on others. Taken together, these data suggest the 2DG exerts anti-inflammatory properties in SA challenged BMDM and BMDN.

### 3.2. 2DG treatment inhibits SA-induced MEK/ERK signaling

Since we observed the downregulation of inflammatory cytokines upon 2DG treatment, we assessed the effect of 2DG on classical MAPKs (p38, JNK and MEK-ERK), involved in inflammatory signaling (Kumar and Shamsuddin, 2012; Singh and Kumar, 2015). Our results show that *S. aureus* induced time-dependent phosphorylation of ERK1/2 in BMDMs and 2DG pretreatment reduced this effect (Fig. 3A). However, 2DG had no effect on p38 and JNK signaling (data not shown). To further validate the effect of 2DG on the downregulation of ERK1/2 phosphorylation, we used a specific pharmacological inhibitor of MEK, U0126. We found that both 2DG, as well as U0126, downregulated the ERK phosphorylation (Fig. 3B), indicating that 2DG behaves similarly to MEK inhibitor. To investigate the biological consequence of 2DG and U0126 mediated downregulation of ERK1/2 phosphorylation, we quantified the production of inflammatory cytokines by ELISA. Our data show that while both 2DG and U0126 treatment significantly reduced IL-1 $\beta$ , and IL-6 levels (Fig. 3C), only 2DG was found to reduce the production of TNF- $\alpha$  and CXCL1, whereas U0126 had no effect, indicating the effect of 2DG on other parallel mechanisms independent of ERK inhibition (Fig. 3C). The levels of CXCL2 remained unchanged and were comparable in both of these drug treatments. The drug alone (without SA infection) had no effect on these cytokines/chemokines production as their levels were similar to uninfected controls.

### 3.3. 2DG treatment attenuates inflammation in staphylococcal endophthalmitis

To validate our *in vitro* findings, we tested the effect of 2DG in a mouse model of *S. aureus* endophthalmitis (Singh et al., 2014a; Talreja et al., 2015). Since we observed the downregulation of ERK1/2 phosphorylation following 2DG pretreatment in cultured cells, we treated mice with 2DG by intravitreal injections 12–14 h prior to SA infection and investigated its effect on intraocular inflammation. Our results show that 2DG treatment significantly reduced the expression of inflammatory mediators, IL-1 $\beta$ , IL-6, CXCL1, and CXCL2 in SA infected eyes, as measured by ELISA (Fig. 4A). To determine whether 2DG inhibits ERK1/2 phosphorylation *in vivo*, western blot analysis was performed on retinal tissue lysate which showed that 2DG treated eyes had reduced ERK1/2 phosphorylation (Fig. 4B). Surprisingly, 2DG had no significant effect on bacterial burden in the eye albeit slight reduction (Fig. 4C).

Because our pretreatment (prophylactic) approach was found reduce intraocular inflammation, we next sought whether 2DG can exert anti-inflammatory effects when given post-infection. Indeed, administered of 2DG after 6h of SA challenge was found to reduce inflammation. Our data show that 2DG post-treatment reduced corneal haze and anterior chamber opacity in mouse eyes (Fig. 5A) but had no significant impact on bacteria growth (Fig. 5B). ERG analysis showed reduction of both a- and b-wave amplitudes in SA infected eyes and 2DG treatment was found to better retain b- but not the a-wave amplitude (Fig. 5C). Most importantly, similar to pre-treatment, 2DG post-treatment reduced pro-inflammatory cytokines/chemokines both at transcript (Fig. 5D) as well as protein levels with the exception of TNF- $\alpha$  (Fig. 5E). Collectively, these results indicate that 2DG treatment pre- or post- SA infection attenuates inflammatory response in endophthalmitis.

#### 4. Discussion

The concept of immunometabolism has greatly evolved in past few years to study the interface between metabolism and immunity as it has provided novel therapeutic targets (O'Neill, L.A., 2016). Because several studies, including those from our laboratory, have implicated the role of glycolysis in promoting inflammation (Hackett et al., 2020; Kumar et al., 2016; Lin et al., 2020; Varanasi et al., 2017; Wei et al., 2020), we investigated the link between glycolytic metabolism and inflammation in intraocular infection, which is currently not known. Here, we demonstrated that inhibition of glycolysis exerts anti-inflammatory effects in macrophages and neutrophils challenged with live or heat killed *S. aureus*. Additionally, 2DG treatment either pre- or post-bacterial infection resulted in diminished intraocular inflammation in experimental staphylococcal endophthalmitis. Mechanistically, 2DG exerted its anti-inflammatory effects via inhibition of bacterial-induced MEK-ERK1/2 pathways (Fig. 6). Collectively, our study suggests that glycolytic inhibitors such as 2DG might aid in controlling excessive inflammation during intraocular infection and can be used as adjunct therapy with antibiotics.

The eye is classically viewed as an immune-privileged organ (Guest et al., 2018; Miller et al., 2019; Mursalin et al., 2020). However, this immune-privileged status can be breached by pathogens which causes damage to ocular tissues, potentially leading to vision impairment or blindness. Various pathogens, including bacteria, viruses, and fungi, have been shown to infect the eye through multiple means including trauma, ocular surgeries, or endogenously through the bloodstream (Gupta et al., 2019; Mursalin et al., 2020). Following infection, retinal residential cells viz. astrocytes, microglia, Müller cells-become activated through recognition of PAMPs via PRR/TLRs activation and induced an innate immune response (Kumar et al., 2013; Singh et al., 2014b; Talreja et al., 2015). Cytokines/chemokines produced by these cells promote the permeabilization of the blood-retinal barrier, attract PMNs and macrophages, and clear the invading pathogens (Kumar and Kumar, 2015; Novosad and Callegan, 2010). However, the persistent influx of innate immune cells and intraocular inflammation leads to the destruction of ocular tissues (Miller et al., 2019; Parkunan et al., 2016; Talreja et al., 2015). Therefore, immunomodulatory therapies are needed to minimize the pathological consequences of excessive inflammation (Miller et al., 2019).

The effects of 2DG (glycolysis inhibition) has been primarily studied in cancer and T-cell mediated diseases (Ciavardelli et al., 2014; Zhang et al., 2006), under conditions known to have high metabolic demand (Chang et al., 2013; Chang et al., 2015; O'Neill et al., 2016). Outcomes of glycolytic inhibition as a treatment in infectious disease models have varied depending on organ/tissue infected, as well as the pathogen involved (Ogawa et al., 1994; Sutrina et al., 2016; Varanasi et al., 2017; Wickersham et al., 2017), implying 2DG efficacy is disease dependent. To test whether 2DG modulates innate responses in bacterial endophthalmitis, we first tested its effect on cytokine production using primary bone marrow-derived macrophages and neutrophils, as these particular innate cells infiltrate the eye soon after bacterial invasion and promote intraocular inflammation. Our data shows that 2DG pretreatment significantly attenuates both live and heat-killed *S. aureus* in BMDM. Our finding corroborated with reports where 2DG has been shown to decrease *S. aureus* induced TNF- $\alpha$  and IL-1 $\beta$  production in keratinocytes (Wickersham et al., 2017), and LPS induced IL-6 production by human macrophages (Chiba et al., 2017) and microglia (Fodelianaki et al., 2019).

Polymorphonuclear neutrophils (PMN) are also known to rely on glycolysis for their effector functions (Loftus and Finlay, 2016) and are essential for bacterial clearance in endophthalmitis (Coburn et al., 2018; Parkunan et al., 2015; Singh et al., 2014a; Talreja et al., 2015). When we inhibited glycolysis using 2DG, the BMDM demonstrated reduced cytokine production following SA challenge. Similarly, 2DG differentially modulated the expression of inflammatory mediators following the HKSA challenge. These results suggest that immune cells, to varying degrees, utilize glycolysis for pro-inflammatory cytokine secretion that can be reduced when blocking the glycolytic pathway. We also observed a slight discrepancy in the reduction of inflammatory cytokine/ chemokines by 2DG in BMDM and BMDN suggesting glycolysis independent expression of some of these inflammatory mediators. Our findings are further supported by studies showing 2DG significantly reduced LPS-induced release of IL-6 and IL-1 $\beta$  in microglia, although it did not influence TNF- $\alpha$  expression suggesting TNF- $\alpha$  expression is regulated by LPS independently of glycolysis in microglia (Fodelianaki et al., 2019). These differential effects of 2DG specific cytokines or chemokines could be harnessed to modulate the pathogenic versus protective effects of these inflammatory mediators in the context of a disease condition.

To determine the *in vivo* relevance of our findings, we tested the anti-inflammatory effect of 2DG in a mouse model of bacterial endophthalmitis (Talreja et al., 2015). We observed that intravitreal injection of 2DG followed by infection with *S. aureus* resulted in a significant reduction in intraocular inflammation by inhibiting the IL-1 $\beta$ , IL-6, CXCL1, and CXCL2 production. We did not observe any effect of 2DG on TNF- $\alpha$  production. Our finding corroborated with a previous study where 2DG treatment was shown to reduce *S. aureus* induced cytokines production, including IL-1 $\beta$ , but had no effect on bacterial burden or PMN infiltration in a skin infection model (Wickersham et al., 2017). Because we also observed no significant reduction in intraocular bacterial growth, the observed anti-inflammatory effects of 2DG are likely due to its ability to inhibit bacterial-induced inflammatory signaling (MEK/ERK) in infiltrated innate immune cells (e.g., PMNs,



macrophages). However, further studies are needed to assess the effect of 2DG on cellular infiltration in bacterial endophthalmitis.

Although 2DG treatment was not found to exhibit significant effect on bacterial burden in infected mouse eyes, some *in vitro* studies have shown that 2DG attenuates the multiplication of intracellular pathogens such as *Legionella pneumophila*, in part via inducing autophagy (Matsuda et al., 2009; Ogawa et al., 1994). We also performed RNASeq analysis of BMDMs challenged with SA in presence or absence of 2DG. Our unpublished data shows induced expression of genes related to autophagy and oxidative stress pathways. However, further validation and more in-depth investigations are needed to draw conclusion on whether 2DG modulates phagocytosis and/or intracellular killing on *S. aureus* by innate immune cells.

It is well known that in infectious conditions, following recognition of PAMPs via TLRs, induction of multiple signaling pathways occurs, including MAPK/ERK kinase (MEK) and NF- $\kappa$ B resulting production of inflammatory mediators (Fig. 6). Because these pathways have been shown to modulate cellular metabolism, including glycolysis (Sanin et al., 2015; Traves et al., 2012), we assessed the effect of 2DG on MAPK signaling pathways. Similar to a previous study (Chiba et al., 2017), we found that 2DG mainly inhibits bacterial-induced ERK pathway and had no significant effect on other MAPKs, such as p38 and JNK (data not shown). Hence our data imply that 2DG exerts its anti-inflammatory effects by inhibiting bacterial-induced ERK resulting in reduced transcription and translation of inflammatory mediators. This observation was further supported by the head-to-head comparison of 2DG with an ERK specific inhibitor, U0126. While our data show that both 2DG and U0126 decreased ERK phosphorylation to similar degrees in BMDMs and 2DG inhibited TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and CXCL1 production, whereas U0126 only inhibited IL-1 $\beta$  and IL-6 production. These findings indicate that 2DG-mediated abrogation of cytokine/chemokine production is not exclusively dependent on ERK inhibition. Recently, we showed that 2DG induces AMPK (Singh et al., 2020), a negative regulator of inflammation, whose activity is reduced in bacterial endophthalmitis (Kumar et al., 2016). Therefore another potential anti-inflammatory mechanism of 2DG is the activation of AMPK.

In summary, our study demonstrates that glycolytic inhibitor, 2DG can limit *S. aureus* induced inflammation in bacterial endophthalmitis by attenuating inflammatory mediators produced by infiltrating macrophages and neutrophils. Additionally, we show that 2DG exerts its anti-inflammatory effects by inhibiting the ERK signaling. As our study establishes the link between cellular metabolism and innate immune cell effector functions, we propose that metabolic inhibitors can be used as an adjunct therapy along with antibiotics to attenuate excessive inflammation in ocular or non-ocular infectious diseases.

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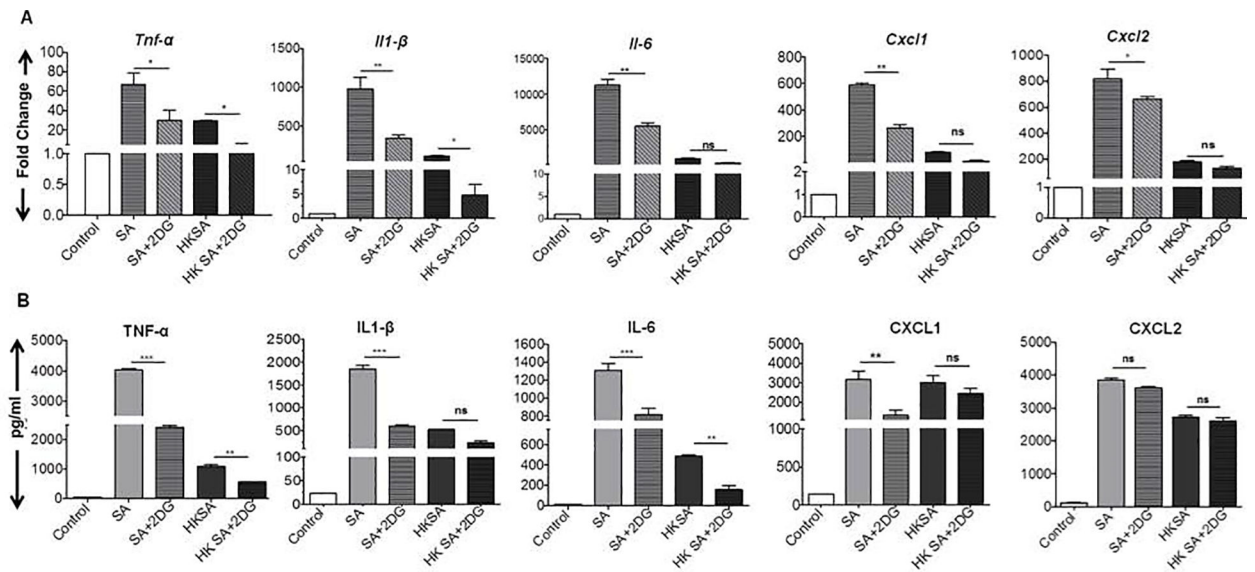
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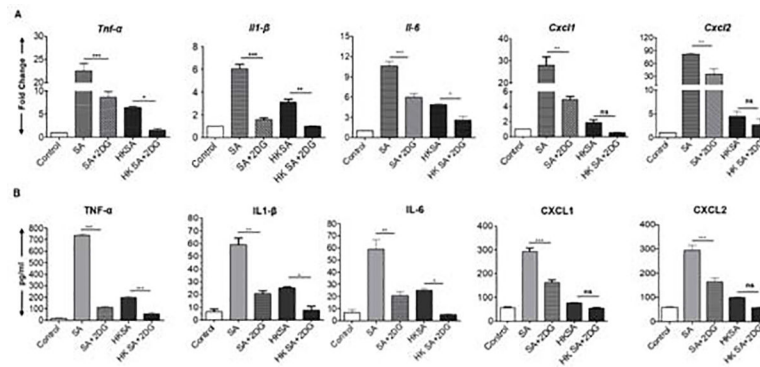
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### Highlights

- First report investigating the role of glycolysis in *S. aureus* (SA) endophthalmitis.
- Glycolytic inhibitor, 2DG, diminished the SA-induced inflammation in BMDM and BMDNs.
- 2DG treatment reduced intraocular inflammation in SA-infected mouse eyes.
- The anti-inflammatory effects of 2DG were mediated by the MEK/ERK pathway inhibition.



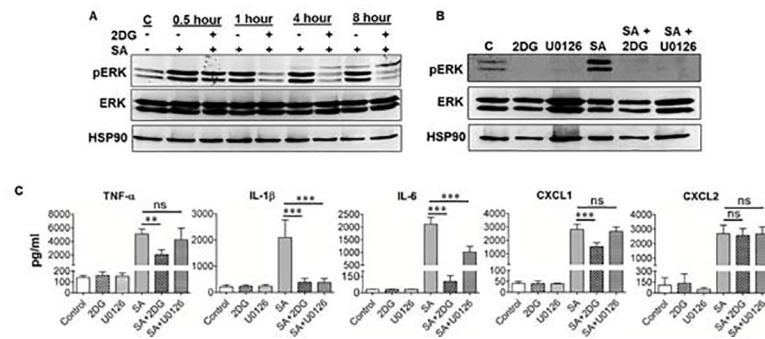
**Figure 1. 2DG treatment attenuates *S. aureus* induced inflammation in the macrophages.** Bone marrow-derived macrophages (BMDMs) were pretreated with 2DG (10 mM) for 1 h followed by challenge with either live *S. aureus* (SA) or heat-killed *S. aureus* (HKSA) for 8 h. Uninfected cells were used as control. Control, SA, and HKSA challenged cells were subjected to qRT-PCR for inflammatory mediators (A). Conditioned media were subjected to ELISA to quantify the cytokine protein levels (B). Data represent mean  $\pm$  SD from three independent experiments. A one-way ANOVA with Tukey post hoc test was used to determine statistical significance. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns: not significant.



**Figure 2. 2DG treatment attenuates SA induced inflammation in the neutrophils.**

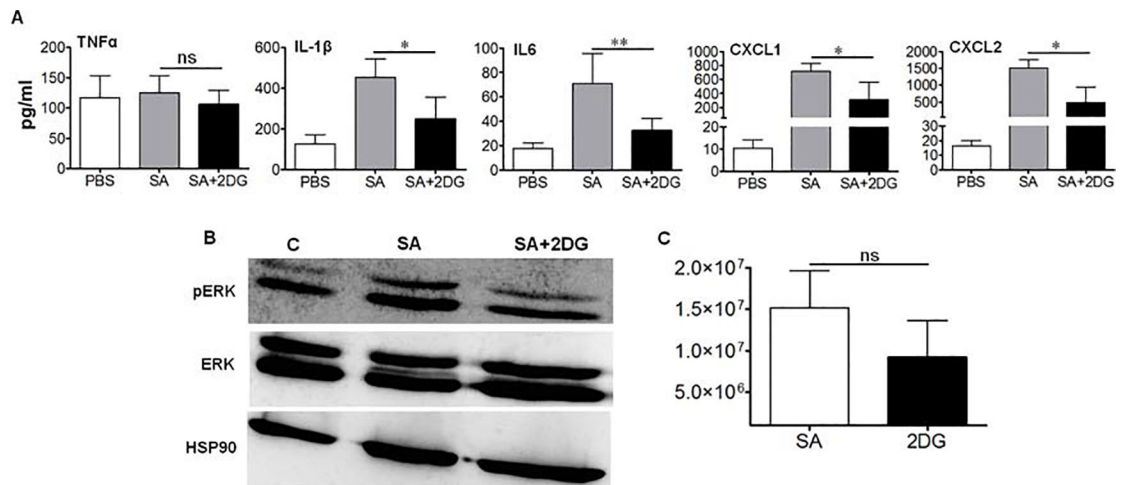
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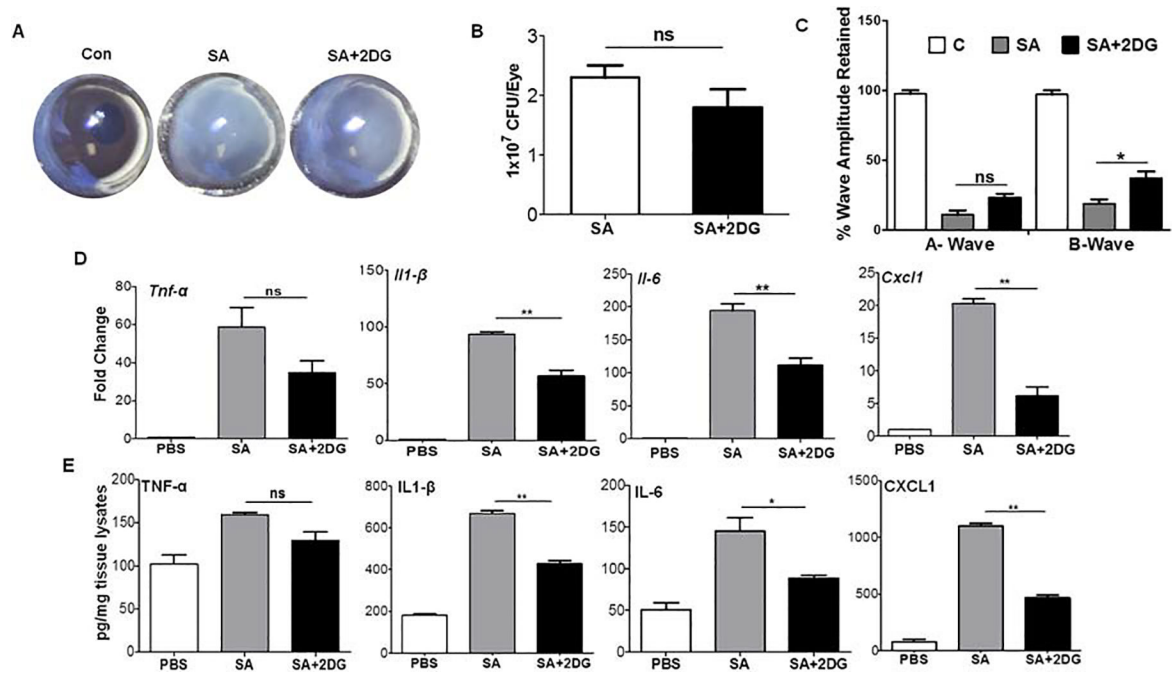
**Figure 3. *S. aureus* induced MEK/ERK pathways is inhibited by 2DG.**

BMDMs were pretreated with 2DG (10 mM) for 1 h followed by challenge with *S. aureus* (SA) for indicated time-points. Cells were harvested and subjected to Western blotting for pERK, ERK and HSP90 proteins (A). BMDMs were pretreated with either 2DG (10 mM) or ERK inhibitor U0126 (10  $\mu$ M) for 1 h, followed by a challenge with SA for 8 h. Cells were harvested and subjected to Western blotting for pERK, ERK and HSP90 proteins (B). The conditioned media from 2DG and U0126 pretreated cells in the presence and absence of SA were subjected to ELISA (C). Data represent mean  $\pm$  SD from three independent experiments. A one-way ANOVA with Tukey post hoc test was used to determine statistical significance. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns: not significant.



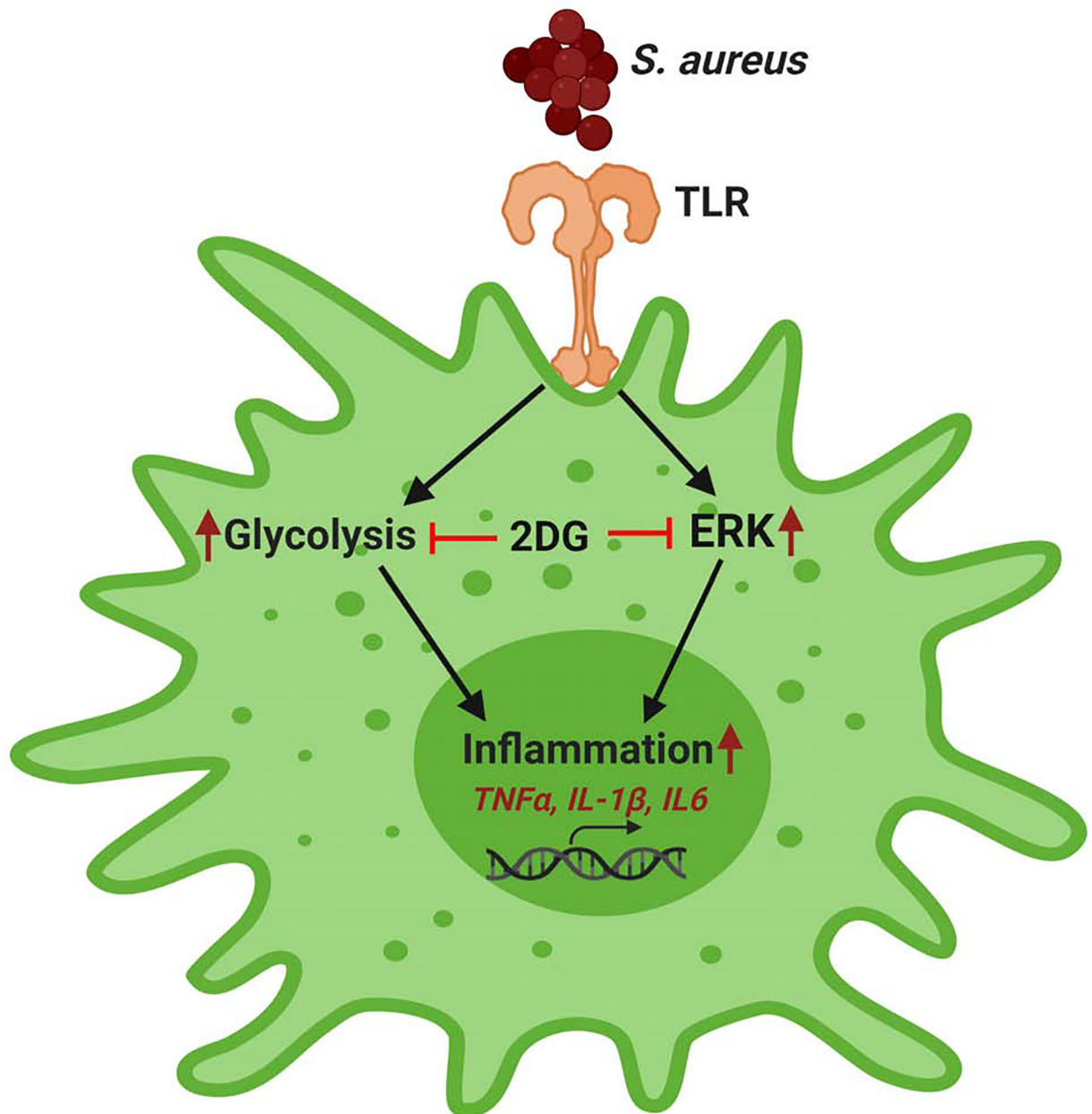
**Figure 4. 2DG pre-treatment reduces intraocular inflammation in *Staphylococcal* endophthalmitis.**

WT C57BL/6 mice (n=10) were pretreated overnight with intravitreal injections of 2DG (25  $\mu$ g/eye) followed by intravitreal injection of *S. aureus*. 24 h post-infection eyes were enucleated, homogenized and subjected to ELISA for cytokine quantification (A), Western blotting for pERK1/2, ERK1/2 and HSP90 proteins (B), and bacterial burden estimation (C). A one-way ANOVA with Tukey post hoc test was used to determine statistical significance. \*\*p<0.01, \*\*\*p<0.001, ns: not significant. Data represent mean  $\pm$  SD.



**Figure 5. 2DG post-treatment attenuates *S. aureus* induced intraocular inflammation in mouse eyes.**

WT C57BL/6 mice (n=10) eyes were infected with *S. aureus* and at 6 h post-infection eyes were treated with 2DG (25  $\mu$ g/eye) by intravitreal injection. 24 h post-2DG treatment eyes were examined and photomicrograph was taken (A). Eyes were enucleated and subjected to bacterial burden estimation by plate count (B). The retinal function was measured using scotopic ERG (C). The retinal tissue was subjected to qPCR for various pro-inflammatory mediators (D). The eye lysates were subjected to ELISA for cytokine/chemokine quantification (E). A one-way ANOVA with Tukey post hoc test was used to determine statistical significance. \*p<0.05, \*\*p<0.01, ns: not significant. Data represent mean  $\pm$  SD.



**Figure 6. A schematic of anti-inflammatory mechanisms of 2DG.**

During bacterial endophthalmitis, *S. aureus* is recognized by TLR2 expressed on innate immune cells, leading to activation of glycolytic and inflammatory (ERK) signaling cascade resulting in induced expression of inflammatory mediators. 2DG exerts anti-inflammatory effects by inhibiting these cellular responses. This schematic was created using BioRender software.