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1 **Myeloid cell-specific deletion of AMPK α 1 worsens ocular bacterial infection by**
2 **skewing macrophage phenotypes**

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22

23 **Abstract**

24 AMP-activated protein kinase (AMPK) plays a crucial role in governing essential cellular
25 functions such as growth, proliferation, and survival. Previously, we observed increased
26 vulnerability to bacterial (*S. aureus*) endophthalmitis in global AMPK α 1 knockout (KO)
27 mice. In this study, we investigated the specific involvement of AMPK α 1 in myeloid cells
28 using LysM^{Cre}; AMPK α 1^{fl} mice. Our findings revealed that while endophthalmitis resolved
29 in wild-type (WT) B6 mice, the severity of the disease progressively worsened in
30 AMPK α 1 deficient mice over time. Moreover, the intraocular bacterial load and
31 inflammatory mediators (e.g., IL-1 β , TNF- α , IL-6, and CXCL2) were markedly elevated
32 in the LysM^{Cre}; AMPK α 1^{fl} mice. Mechanistically, the deletion of AMPK α 1 in myeloid cells
33 skewed macrophage polarization toward the inflammatory M1 phenotype and impaired
34 the phagocytic clearance of *S. aureus* by macrophages. Notably, transferring AMPK-
35 competent bone marrow from wild-type mice to AMPK α 1 KO mice preserved retinal
36 function and mitigated the severity of endophthalmitis. Overall, our study underscores
37 the role of myeloid-specific AMPK α 1 in promoting the resolution of inflammation in the
38 eye during bacterial infection. Hence, therapeutic strategies aimed at restoring or
39 enhancing AMPK α 1 activity could improve visual outcomes in endophthalmitis and other
40 ocular infections.

41

42

43 **Keywords:** Eye, Endophthalmitis, *S. aureus*, Inflammation, AMPK,
44 Immunometabolism, macrophages

45

46 **Introduction**

47 Bacterial endophthalmitis is a serious eye infection that can lead to vision loss
48 due to severe intraocular inflammation and tissue damage [1-4]. Staphylococci,
49 especially *Staphylococcus (S) aureus*, are the primary culprits, and although they
50 account for only 10-15% of cases, they often lead to poor outcomes [5]. Typically,
51 during ocular surgeries, *S. aureus* gains entry into the eye, releasing virulence factors
52 such as toxins and cell wall components such as peptidoglycan and lipoteichoic acids
53 [6]. Host immune cells recognize these bacterial elements through pattern recognition
54 receptors and trigger a complex inflammatory immune response [3, 7]. Moreover, the
55 blood-retinal barrier, which is crucial for ocular defense, becomes compromised,
56 allowing a large influx of immune cells, predominantly neutrophils and
57 monocytes/macrophages, into the vitreous chamber [8, 9]. While this influx is necessary
58 to combat bacterial growth, it also leads to excessive inflammation, which, if unchecked,
59 damages intraocular tissues, especially the neurosensory retina, resulting in irreversible
60 vision loss [2, 6, 10]. Unfortunately, there is no standardized treatment to control
61 intraocular inflammation, and visual outcomes often remain suboptimal even after
62 aggressive antibiotic treatments and vitrectomy surgeries [11, 12]. Moreover, the
63 increase in antibiotic-resistant ocular pathogens poses a significant challenge in
64 managing these infections [13, 14]. Therefore, research aimed at understanding the
65 mechanisms governing inflammation initiation and resolution holds promise for
66 identifying potential therapeutic targets to prevent and treat bacterial endophthalmitis.

67 Given the intricate nature of host-pathogen interactions, we employed an
68 integrated approach combining metabolomics and transcriptome analysis of *S. aureus*-

69 infected retina tissues to identify novel immunomodulatory and antimicrobial targets [15-
70 17]. Our findings revealed significant impacts on various pathways during
71 endophthalmitis, including inflammatory and immune responses, antimicrobial defense
72 mechanisms against bacteria, endoplasmic stress, cell trafficking, and death, as well as
73 energy metabolism, notably involving disruptions in NAD⁺ and lipid biosynthesis [1, 15,
74 16, 18]. Within the realm of energy metabolism, AMP-activated protein kinase (AMPK)
75 has emerged as a key player in maintaining host energy balance [19]. AMPK not only
76 governs cellular energy metabolism but also regulates immune responses by
77 modulating immune signaling pathways [20-22]. This cross-talk between AMPK and
78 immune signaling pathways has crucial implications for reshaping the metabolic and
79 functional profiles of immune cells [23, 24].

80 In our previous work, we demonstrated that both genetic (using AMPK α 1 global
81 knockout mice) and pharmacological (with AICAR) activation of AMPK confer protection
82 against endophthalmitis [1]. However, a limitation of this study was the inability to
83 discern the cell-specific contribution of AMPK to the pathogenesis of bacterial
84 endophthalmitis. Given that the primary immune cells involved in endophthalmitis are of
85 myeloid origin, we aimed to investigate the specific role of AMPK α 1 in myeloid cells
86 during endophthalmitis. In this study, employing myeloid-specific AMPK α 1 knockout
87 mice (LysM^{Cre}; AMPK α 1^{fl}) and bone marrow reconstitution chimera approaches, we
88 found that the loss of AMPK α 1 in myeloid cells worsens bacterial endophthalmitis
89 highlighting the importance of myeloid-specific AMPK in bacterial endophthalmitis.

90 **Materials and Methods**

91 **Mice and ethical statement**

92 B6.SJL-Ptprca Pepcb/BoyJ (Pep Boy) mice were obtained from the Jackson
93 Laboratory (Bar Harbor, ME), while wild-type (WT) and AMPK α 1 global knockout (KO)
94 (B6/129 background) were used as described [1]. AMPK α 1^{fl} mice were purchased from
95 Jackson laboratory (#014141) and crossed with LysM^{Cre} (#004781) to generate a
96 myeloid-specific loss of AMPK α 1 referred to as LysM^{Cre}; AMPK α 1^{fl}. Six- to eight-week-
97 old mice of both male and female sex were utilized. They were housed in a controlled-
98 access Division of Laboratory Animal Resources (DLAR) facility at the Kresge Eye
99 Institute, maintained under a 12-hour light/12-hour dark cycle at a temperature of 22°C,
100 and provided ad libitum access to water and LabDiet rodent chow (PicoLab; LabDiet, St.
101 Louis, MO). All procedures were conducted per the Association for Research in Vision
102 and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision
103 Research and were approved by the Institutional Animal Care and Use Committee
104 (IACUC) of Wayne State University (protocol # IACUC-22-04-4557).

105 **Bacterial strain and induction of endophthalmitis**

106 The *S. aureus* strain RN6390 was cultured in tryptic soy broth and on agar plates
107 (TSB/TSA; Sigma-Aldrich, St. Louis, MO). Bacterial endophthalmitis was induced in
108 mice, as described previously [3, 25]. Briefly, mice were anesthetized and intravitreally
109 injected with *S. aureus* (500 CFU/eye) using a 34G needle under a dissecting
110 microscope within a biosafety cabinet. Contralateral eyes received sterile PBS injections
111 and served as controls. Disease progression was monitored through slit lamp
112 examination and other modalities. Scotopic electroretinography (ERG) was used to
113 determine retinal function using the Celeris ERG system (Diagnosis LLC, Lowell, MA),

114 as per the manufacturer's recommendation and standardized in our prior studies [16,
115 26].

116 **Isolation of bone marrow-derived macrophages (BMDMs)**

117 Mouse bone marrow-derived macrophages (BMDMs) were isolated according to
118 previously established protocols [27]. Briefly, mice were euthanized, and bone marrow
119 was flushed from the tibias and femurs using RPMI media supplemented with 10% FBS
120 and 0.2 mM EDTA. The cells were then centrifuged at 400xg for 5 minutes at 4°C to
121 pellet them. Red blood cells (RBCs) were lysed by adding 0.2% NaCl (hypotonic)
122 solution for 20 seconds, followed by the addition of 1.6% NaCl (hypertonic) and
123 subsequent centrifugation. The cell pellets were rinsed with RPMI media, re-suspended,
124 seeded in RPMI media supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/mL
125 streptomycin, and 10 ng/ml M-CSF for macrophage differentiation and cultured at 37°C
126 in 5% CO₂. Six days post-differentiation, BMDMs were seeded at a concentration of
127 4×10^6 cells/mL in 60 mm Petri dishes for *in vitro* experiments.

128 **Bacterial burden estimation**

129 The eyes were enucleated following *S. aureus* infection at 24 h, 48 h, and 72 h
130 post-infection. Whole-eye lysates were prepared in 250 µL of PBS by beating with
131 stainless steel beads in a tissue lyser (Qiagen, Valencia, CA). A 50 µL aliquot of the
132 tissue homogenate was serially diluted and plated on Tryptic Soy Agar (TSA) to quantify
133 the viable bacterial load. The colonies were counted the next day, and the results are
134 reported as the mean number of CFU/eye.

135 **Cytokine ELISA**

136 For *in-vivo* samples, eyes were enucleated, and lysates were prepared using a
137 tissue lyser as described above. The tissue homogenates were then centrifuged at
138 10,000 x g for 20 minutes at 4°C, and the resulting supernatant was utilized for cytokine
139 estimation. In the *in vitro* studies, conditioned media from both the *S. aureus*-infected
140 and drug-treated groups, as well as the vehicle control group, were collected and
141 preserved for cytokine measurements. Mouse ELISA was conducted to quantify the
142 inflammatory cytokines IL-1 β , IL-6, TNF- α , and CXCL2 according to the manufacturer's
143 instructions (R&D Systems, Minneapolis, USA).

144 **Histological analysis**

145 The eyes were enucleated and preserved in modified Davidson's fixative for
146 subsequent histopathological analysis. The embedding, sectioning, and staining with
147 hematoxylin & eosin (H&E) were carried out by Excalibur Pathology, Inc. (Oklahoma
148 City, OK, USA).

149 **RNA extraction and qPCR analysis**

150 Total RNA was extracted from both the neural retina and BMDMs using TRIzol
151 according to the manufacturer's guidelines (Thermo Scientific, Rockford, IL).
152 Subsequently, the RNA was reverse transcribed using a Maxima first-strand cDNA
153 synthesis kit following the manufacturer's protocol (Thermo Scientific, Rockford, IL).
154 Quantitative assessment of gene expression was conducted via SYBR green-based
155 qPCR, employing gene-specific primers, on the Step One Plus Real-Time PCR System
156 (Applied Biosystems, Foster City, CA, USA). Data analysis was performed utilizing the
157 comparative $\Delta\Delta$ Ct method, as previously described [28]. The expression levels of genes
158 in the test samples were normalized to endogenous β -actin controls.

159 **Bacterial phagocytosis assay**

160 BMDM cells (10^6 cells/dish) were cultured in 60 mm Petri dishes in RPMI-1640
161 media. Subsequently, the cells were exposed to *S. aureus* at an MOI of 10:1 for 2
162 hours. After the incubation period, the cells were washed and treated with gentamicin
163 (200 $\mu\text{g}/\text{mL}$) for 2 hours to eliminate extracellular bacteria. The absence of extracellular
164 bacteria was confirmed through dilution plating and enumeration of CFUs. Two hours
165 post-gentamicin treatment, the cells were washed with RPMI-1640 and incubated with
166 fresh media containing gentamicin (200 $\mu\text{g}/\text{mL}$) for 1, 3, or 6 hours. To quantify
167 internalized bacteria, following the designated incubation periods, the cells were
168 washed three times with PBS and lysed using 0.01% Triton X-100. The lysed cells were
169 collected by scraping and centrifuged at 7500 x g for 5 minutes. The resulting cell
170 pellets were washed twice with PBS through centrifugation at 7500 x g for 5 minutes
171 each time. The pellets were then resuspended in 1 mL sterile PBS, serially diluted, and
172 plated onto TSA plates for enumeration of bacterial counts.

173 **Immunostaining**

174 BMDMs were cultured in a four-well chamber slide (Fisher Scientific, Rochester,
175 NY). Following treatment, the cells were infected with *S. aureus* at an MOI of 10:1 for 3
176 hours. At the specified time points, the infected cells were fixed with 4% PFA in PBS at
177 4°C. After washing with PBS, the cells were permeabilized and blocked with 1% (w/v)
178 BSA and 0.4% Triton X-100 in PBS for 1 hour at room temperature (RT). Subsequently,
179 the cells were incubated overnight at 4°C with primary antibodies against iNOS and
180 Arg1 (1:100 dilution, Cell Signaling Technology, Danvers, MA). Following the removal of
181 the primary antibodies, the cells were thoroughly washed with PBS and then incubated

182 with anti-mouse/rabbit Alexa Fluor 485/594-conjugated secondary antibodies (1:200
183 dilution) for 1 hour at RT. Finally, the cells were washed four times (5 minutes each)
184 with PBS, and the slides were mounted in Vectashield antifade mounting medium
185 containing DAPI (Vector Laboratories, Burlingame, CA) before visualization using a
186 Keyence microscope (Keyence, Itasca, IL).

187 **Western blotting**

188 Protein samples were obtained by directly lysing cells in
189 radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase
190 inhibitor cocktails. The total protein concentration of the cell lysates was determined
191 using a Micro BCA™ protein assay kit (Thermo Scientific, Rockford, IL). Subsequently,
192 protein samples (30-40 µg) were separated via SDS-PAGE (8% or 12%) and
193 transferred onto a nitrocellulose membrane (0.45 µm) using a wet transfer system. The
194 membranes were then blocked in 5% (w/v) dry milk in Tris-buffered saline with Tween
195 (TBST) for 1 hour at room temperature. Following blocking, the membranes were
196 incubated with primary antibodies against iNOS, Arg1, and HSP-90 (Cell Signaling
197 Technology, Danvers, MA) according to the manufacturer's instructions, followed by
198 incubation with appropriate horseradish peroxidase-conjugated secondary antibodies.
199 After four TBST washes, the membranes were exposed to SuperSignal West Femto
200 chemiluminescent substrate and imaged using an iBright fluorescence imager (Thermo
201 Scientific, Rockford, IL). Densitometry analysis was carried out using ImageJ software
202 (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland)

203 **Flow cytometry**

204 Flow cytometry was conducted following previously established methods to
205 assess the adoptive transfer and infiltration of neutrophils into the eyes [3]. In brief,
206 retinas from euthanized mice were isolated and digested with Accumax (Millipore) for 10
207 minutes at 37°C. A single-cell suspension was generated by gently triturating the cell
208 mixture with a 22G needle and subsequently passing it through a 40-µm cell strainer
209 (BD Falcon, San Jose, CA, USA). The cells were then blocked using Fc Block (BD
210 Biosciences) for 30 minutes, followed by three washes with PBS containing 1% bovine
211 serum albumin (BSA). Subsequently, the cells were stained by incubating them with
212 CD45.1-APC, CD45.2-PE, and Ly6G-FITC conjugated antibodies (BD Biosciences) on
213 ice for 20 minutes in the dark. After incubation, the cells were washed and suspended in
214 PBS supplemented with 1% BSA. Flow cytometric analysis was performed using an
215 Accuri C6 flow cytometer and accompanying software (BD Biosciences), data analysis
216 was conducted using FlowJo™ v8 Software (BD Life Sciences).

217 **Bone marrow chimera**

218 The adoptive transfer of naïve bone marrow cells into AMPKα1 KO mice (with the
219 CD45.2 allele) and B6. SJL-Ptprca Pepcb/BoyJ (Pep Boy, used as wild-type mice with
220 the CD45.1 allele) was carried out via the busulfan method [29]. Initially, recipient mice
221 received a single preconditioning dose of 20 mg/kg busulfan (Cayman Chemicals, Ann
222 Arbor, MI) intraperitoneally daily until a cumulative dose of 100 mg/kg was achieved.
223 Subsequently, all mice were intravenously injected with 1×10^7 donor bone marrow cells
224 obtained from the recipient mouse via tail vein injection in 200 µL of phosphate-buffered
225 saline (PBS). Starting at 2 weeks after transplantation, approximately 20-30 µL of blood
226 was collected from the lateral saphenous vein, and CD45.1 vs CD45.2 expression was

227 assessed in peripheral blood using flow cytometry. Efficient engraftment of donor bone
228 marrow (>80%) was observed at 6 weeks post-transplantation. Following successful
229 engraftment, the mice were subjected to endophthalmitis as described above.

230

231 **RNA sequencing**

232 For RNA sequencing, total RNA was extracted from the BMDMs of the AMPK α 1
233 KO and WT mice in biological triplicates using RNeasy Mini Kits (Qiagen, Hilden,
234 Germany). The sequencing procedures were conducted by Novogene Co., Ltd. RNA
235 sequencing (RNA-seq) libraries were constructed from high-quality RNA using NEBNext
236 Ultra™ II RNA Library Prep kits (New England BioLabs, Ipswich, MA, USA) following
237 the manufacturer's instructions. Sequencing was performed on the NovaSeq 6000
238 platform as paired-end 150-bp reads (Illumina), with a sequencing depth of
239 approximately 30 million reads per sample. Differential gene expression analyses were
240 conducted using DESeq2, while GO enrichment analysis was carried out using
241 PANTHER and KEGG pathways analyses.

242 **Statistical Analysis**

243 Statistical analyses were conducted using Prism version 9.2 (GraphPad, San
244 Diego, CA). Group comparisons were assessed using one-way ANOVA followed by
245 Tukey's Multiple Comparison post-hoc tests, and data were presented as mean \pm SD. A
246 confidence interval of 95% was applied for all statistical tests. A p-value of <0.05 was
247 considered statistically significant. Unless specified otherwise, all experiments were
248 performed at least three times.

249 **Results**

250 ***Myeloid-specific AMPK α 1 deficiency exacerbated bacterial endophthalmitis***

251 The hallmark of bacterial endophthalmitis is the infiltration of myeloid cells such
252 as neutrophils and monocytes into the eye [6]. To investigate the role of AMPK in these
253 cells during ocular infections, we induced *S. aureus* endophthalmitis in both AMPK α 1^{fl}
254 (Wt) and LysM^{Cre}; AMPK α 1^{fl} mice using a low-dose infection model, in which 500
255 colony-forming units (CFUs) of *S. aureus* were administered. Although this dosage
256 resolved in wild-type mice, it led to severe endophthalmitis in LysM^{Cre}; AMPK α 1^{fl} mice
257 [3, 16]. We monitored the progression of endophthalmitis through daily eye exams,
258 retinal function testing, histopathological analysis, and estimation of intraocular bacterial
259 burden and inflammation. Compared to AMPK α 1^{fl} mice, endophthalmitis worsened in
260 LysM^{Cre}; AMPK α 1^{fl} mice, indicated by a time-dependent increase in corneal haze,
261 opacity, and hypopyon formation (**Fig. 1A**). Histopathological analysis revealed
262 increased retinal folding, tissue disintegration, and significant cellular infiltrates in the
263 vitreous chamber of LysM^{Cre}; AMPK α 1^{fl} mouse eyes (**Fig. 1B**). Electroretinography
264 (ERG) demonstrated a notable decrease in both a- and b-wave amplitudes in LysM^{Cre};
265 AMPK α 1^{fl} eyes at various time points (**Fig. 1C**). Additionally, the bacterial burden was
266 relatively greater in LysM^{Cre}; AMPK α 1^{fl} mice (**Fig. 1D**). Overall, a greater percentage of
267 eyes exhibited damage due to *S. aureus* endophthalmitis in LysM^{Cre}; AMPK α 1^{fl} mice
268 compared to AMPK α 1^{fl} (**Fig. 1E**).

269 Furthermore, in addition to direct retinal damage caused by bacterial toxins,
270 uncontrolled inflammation resulting from excessive infiltration and activation of innate
271 immune cells such as neutrophils contributes to bystander tissue damage. To evaluate
272 intraocular inflammation, we analyzed the expression of inflammatory cytokines at both

273 the mRNA (**Fig. 2A**) and protein (**Fig. 2B**) levels at various time points. Notably,
274 myeloid-specific deletion of AMPK α 1 led to higher expression of inflammatory cytokines.
275 For instance, the transcript levels of *Il-6* and *Cxcl2* were elevated at 72 hours, while the
276 protein levels increased at both 48 and 72 hours. Conversely, AMPK α 1-deficient
277 macrophages exhibited consistently increased transcript levels of the inflammatory
278 cytokines *IL-1 β* and *TNF- α* at all time points, with protein levels peaking at 48 or 72
279 hours. Additionally, we investigated the inflammatory response of bone marrow-derived
280 macrophages (BMDMs) from both AMPK α 1^{fl} and LysM^{Cre}; AMPK α 1^{fl} mice, revealing
281 increased levels of inflammatory mediators in AMPK α 1 KO BMDMs consistent with the
282 *in vivo* data (**Fig. S1**). Taken together, these findings suggest that AMPK α 1 deficiency
283 in myeloid cells enhances inflammation and exacerbates endophthalmitis.

284 ***AMPK α 1 deficient BMDMs exhibit distinct transcriptomic profiles***

285 To elucidate the mechanisms underlying the heightened inflammatory response
286 of *S. aureus*-infected AMPK α 1-deficient mice, we performed RNA-seq analysis. This
287 analysis revealed ~3,516 differentially expressed genes (DEGs) in *S. aureus*-infected
288 AMPK α 1 KO BMDMs compared to Wt, with 1,665 genes showing upregulation and
289 1,848 displaying downregulation (**Fig. 3A**). Gene set enrichment analysis indicated
290 significant alterations in pathways associated with inflammation, metabolism,
291 endoplasmic reticulum stress, and cholesterol biosynthesis, with notable downregulation
292 of bacterial defense pathways in AMPK α 1-deficient macrophages (**Fig. 3B**). Further
293 analysis of DEGs based on the encoded proteins corroborated these findings, i.e., a
294 reduction in pathways related to inflammation resolution and responses to
295 lipopolysaccharide and bacteria (**Fig. 3C**). A heat map was constructed to visualize the

296 highly downregulated and upregulated genes involved in inflammatory and antimicrobial
297 immune defense (**Fig. 3D**).

298 Given the increased bacterial burden observed in *S. aureus*-infected $LysM^{Cre}$;
299 $AMPK\alpha1^{fl}$ mice, we hypothesized that this might stem from the downregulation of
300 antimicrobial genes such as *S100a7*, *a8*, and *a10* in the RNAseq data. To verify this, we
301 examined their expression via qPCR and found that their levels were significantly lower
302 in $AMPK\alpha1$ KO BMDMs (**Fig. S2A**). Consistently, $AMPK\alpha1$ KO BMDMs exhibited
303 reduced intracellular phagocytic killing of *S. aureus* in the gentamicin protection assay
304 (**Fig. S2B**). Collectively, these findings underscore the role of $AMPK\alpha1$ in regulating
305 antibacterial activity by enhancing phagocytic killing and the induction of antimicrobial
306 molecules during bacterial infection.

307 ***AMPK α 1* deficiency promotes the M1 milieu during *S. aureus* endophthalmitis**

308 Our RNAseq analysis revealed that a diminished response to bacterial infection
309 corresponds with the exacerbated endophthalmitis observed in $AMPK\alpha1$ -deficient mice.
310 Given the pivotal role of macrophages in inflammation resolution *via* the transition from
311 a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype, we examined the
312 expression of these markers in infected retinal tissue and cultured BMDMs. Our data
313 demonstrated relatively higher transcript levels of the classical M1 markers *Cox2*, *iNos*,
314 and *I/23*, in the retinas of $LysM^{Cre}$; $AMPK\alpha1^{fl}$ mice compared to Wt mice (**Fig. 4A**).
315 Conversely, the levels of M2 phenotype markers (*Arg1*, *Fizz1*, and *Yam1/2*) were
316 downregulated in $LysM^{Cre}$; $AMPK\alpha1^{fl}$ mice retinas. To corroborate these findings, we
317 assessed the pro-inflammatory (M1) and anti-inflammatory (M2) signatures in BMDMs
318 isolated from $LysM^{Cre}$; $AMPK\alpha1^{fl}$ and $AMPK^{fl}$ mice, challenging them with *S. aureus*.

319 Immunostaining of *S. aureus*-infected LysM^{Cre}; AMPKα1^{fl} macrophages revealed
320 increased levels of iNos (**Fig. 4C**) and decreased expression of Arg1 (**Fig. 4D**).
321 Similarly, western blot analysis also demonstrated increased protein levels of iNos (**Fig.**
322 **4E&F**) and decreased Arg1 levels (**Fig. 4G&H**) in AMPKα1-deficient macrophages.
323 These results further support the notion that AMPKα1 regulates the balance between
324 the M1 and M2 phenotypes, whereby its deficiency promotes the inflammatory
325 phenotype in the eye.

326 ***Bone marrow reconstitution of AMPKα1-deficient mice ameliorated***
327 ***endophthalmitis***

328 To illustrate the protective function of myeloid AMPKα1 during endophthalmitis,
329 we used a bone marrow chimera approach, in which bone marrow from donor PepBoy
330 mice (CD45.1 allele) were transferred to recipient AMPKα1-deficient mice (CD45.2
331 allele) (Wt > KO) and vice versa (KO > Wt) (**Fig. 5A- schematic**). This adoptive transfer
332 yielded greater than 80% efficiency of bone marrow transfer in the recipient mice. Once
333 stable bone marrow chimeras were established, *S. aureus* endophthalmitis was
334 induced, and a time-course study was conducted. Eye imaging revealed decreased
335 opacity and corneal haze in the bone marrow of AMPKα1 KO mice with Wt (PepBoy)
336 bone marrow compared to those with AMPKα1 KO bone marrow in Wt mice (**Fig. 5B**).
337 Electroretinography analysis demonstrated better retention of a- and b-wave amplitudes
338 in AMPKα1 KO mice with Wt bone marrow (**Fig. 5C**). Furthermore, transfer of Wt
339 chimera in AMPKα1 KO mice mitigated disease severity, as evidenced by decreased
340 corneal opacity (**Fig. 5D**) and reduced intraocular bacterial burden (**Fig. 5E**).

341 Subsequently, we assessed intraocular inflammation and found that Wt > KO
342 chimera exhibited significantly lower levels of inflammatory markers such as IL-1 β , IL-6,
343 TNF- α , and CXCL2 (**Fig. 5F**). This reduction correlated with a decrease in neutrophil
344 infiltration in AMPK α 1 KO chimera (**Fig. 5G**) compared to PepBoy mice with AMPK α 1-
345 deficient bone marrow (**Fig. 5H**). Thus, myeloid-specific AMPK α 1 contributes to
346 inflammation resolution and mitigates bacterial endophthalmitis.

347

348 **Discussion**

349 Bacterial endophthalmitis remains the most common vision-threatening
350 complication of eye surgeries and ocular trauma [4]. Owing to the rapid progression and
351 poor prognosis of endophthalmitis, early treatments with intravitreal injections of
352 empirical antibiotics remain the main treatment option for bacterial endophthalmitis [30].
353 Antibiotics, while destroying bacteria, may release lipopolysaccharides [31, 32]
354 lipoteichoic acid, and peptidoglycan from bacterial cell walls, including that of *S. aureus*
355 (SA)[32], and evoke an inflammatory response [2, 33]. To suppress inflammation,
356 intravitreal injections of corticosteroids are recommended, but the usage of steroids
357 along with antibiotics for endophthalmitis has been marred with controversial outcomes
358 in humans [34-38] and animal studies [39-43]. Moreover, steroid-related side effects,
359 including elevated intraocular pressure (IOP), cataract formation, and retinal
360 detachment, further hamper their inclusion in the management of endophthalmitis [44].
361 Thus, there is an urgent need to develop non-immunosuppressive anti-inflammatory
362 therapies. In this study, we demonstrated an anti-inflammatory and protective role of
363 myeloid-specific AMPK in bacterial endophthalmitis.

364 AMPK is an evolutionarily conserved heterotrimeric kinase with α , β , and γ
365 subunits that primarily regulate cellular energy homeostasis [23]. The AMPK complex is
366 activated either by increases in cellular AMP or ADP or by phosphorylation on Thr172
367 by upstream kinases, such as CaMKK- β , LKB1, or TAK1 [45-50]. This enhanced
368 activity, in turn, controls signaling pathways that regulate protein synthesis, cell division,
369 and intracellular membrane trafficking. Recent studies on AMPK regulation indicate that
370 it not only functions as an intracellular energy sensor but also functions as a stress
371 sensor to maintain intracellular homeostasis during infectious, autoimmune diseases
372 and skeleton muscle regeneration [51-53]. Previously, using global AMPK α 1 KO mice,
373 we reported that loss of AMPK α 1 increased the severity of bacterial endophthalmitis.
374 However, the cell-specific role of AMPK α 1 was not determined. Here, our data showed
375 that myeloid cell-specific AMPK α 1 plays a protective role during endophthalmitis as
376 evidenced by worse disease outcomes in LysM^{Cre}; AMPK α 1^{fl} mice (**Fig. 6**). Moreover, the
377 protective effects of AMPK α 1 on myeloid cells are exhibited via several potential
378 mechanisms.

379 Here, we observed that the bacterial burden was greater in myeloid-specific
380 AMPK α 1 deficient mice. As we reported that activation of AMPK α and its downstream
381 ACC in retinal Müller glia regulate autophagy-mediated clearance of *S. aureus* [17], we
382 hypothesized that lack of AMPK α will impair pathogen clearance. Notably, phagocytosis
383 of the pathogen activates the host autophagy initiation complex and the upstream
384 regulatory components LKB1 and AMPK α 1, which regulate autophagy induction through
385 their kinase activities [54]. Deletion of AMPK α 1 in myeloid cells results in susceptibility
386 to exacerbated bacterial infection and host tissue cell death. In support of these

387 findings, our data showed that BMDMs from $LysM^{Cre}; AMPK\alpha1^{fl}$ mice had reduced
388 phagocytic killing of *S. aureus*. Moreover, AMPK α 1 deficiency led to a significant
389 decrease in the bacterial infection-induced expression of antimicrobial peptides which
390 might also contribute to greater bacterial burden in the eyes. Our results corroborate
391 previous studies reporting the increased phagocytic activity (*E. Coli* and *L.*
392 *pneumophila*) of macrophages upon pharmacological activation of AMPK *via* AICAR or
393 metformin treatment [55, 56]. Moreover, the defect in phagocytosis following AMPK α 1
394 ablation in macrophages is associated with their inability to undergo phenotypic
395 transition, explaining the defect in bacterial clearance. A recent study has shown that
396 restoring AMPK activity accelerates recovery from bacterial pneumonia [57]. Similarly,
397 we also reported that AMPK activation inhibits viral replication in retinal cells [58]. These
398 studies highlight the multifaceted roles of AMPK in viral and bacterial infections [59]
399 through the regulation of cellular metabolism [60, 61].

400 Macrophages are also highly plastic innate immune cells, and environmental
401 stimuli or bacterial infection can generate macrophages with a range of different
402 phenotypes and functions [62]. The classical activation of macrophages generates M1
403 macrophages with pro-inflammatory properties, while alternative activation generates
404 M2 macrophages with anti-inflammatory properties [63]. M1 macrophages exacerbate
405 tissue injury whereas M2 macrophages promote the resolution of inflammation upon
406 tissue injury [63, 64]. A careful examination of $LysM^{Cre}; AMPK\alpha1^{fl}$ revealed impaired
407 inflammation resolution, leading to a subtle but significant increase in the expression of
408 M1 macrophage phenotype markers (*iNos* and *Il23* expression) with the endophthalmitis
409 progression. In contrast, the same mice showed a decrease in M2 macrophage

410 phenotype markers (*Arg1* and *Fizz1*), corresponding to an anti-inflammatory state,
411 resulting in accelerated inflammation and endophthalmitis. Interestingly, a previous
412 study showed that AMPK α 1 mediated the regulation of macrophage skewing at the time
413 of inflammation resolution during skeletal muscle regeneration [53]. Our data showing
414 higher levels of inflammatory mediators in LysM^{Cre}; AMPK α 1^{fl} mice eyes indicate the
415 predominance of the M1 milieu which results in inflammation mediated retinal tissue
416 damage.

417 To further confirm the role of AMPK α 1 in ocular infection, we generated bone-
418 marrow chimeras and showed the ability of AMPK α 1-competent bone marrow-derived
419 cells to rescue AMPK α 1 KO from severe endophthalmitis. Consequently, impaired
420 inflammation resolution was observed in KO -> Wt chimera where Wt macrophages
421 were replaced with macrophages lacking AMPK α 1 expression, whereas a benefit was
422 observed in Wt -> KO chimera expressing AMPK α 1 in macrophage. Moreover, the
423 extent of endophthalmitis resolution in the AMPK α 1 KO recipients was similar to those
424 in Wt mice. This indicated that AMPK α 1 expressed in infiltrating/residential myeloid cells
425 contributes to endophthalmitis resolution.

426 In summary, our study demonstrated the protective role of myeloid cell-specific
427 AMPK α 1 during bacterial endophthalmitis. These effects are mediated by enhanced
428 phagocytic clearance of bacteria and the promotion of inflammation resolution via
429 inducing M2 macrophage phenotype. Therefore, AMPK α 1 activation can be targeted
430 therapeutically to treat bacterial endophthalmitis.

431

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440

441 **Conflict of Interest.**

442 The authors declare no competing interests.

443 **Data availability**

444 All data are contained within the article.

445

446 **References**

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604 **Figure Legends**

605 **Fig. 1: Myeloid-specific AMPK α 1 deficiency exacerbates endophthalmitis severity**

606 **in mice. (A-B)** Endophthalmitis was induced in AMPK α 1^{fl} (Wt mice) or LysM^{Cre}; α 1^{fl}
607 (myeloid-specific AMPK α 1 deficient) n = 6 per time point: 24, 48, and 72 h) by
608 intravitreal inoculation of *S. aureus* RN6390 (5,00 CFUs/eye). Eyes injected with PBS
609 were used as controls. Representative slit-lamp micrograph showing corneal
610 haze/opacity and H&E staining of enucleated eyes showing retinal damage and immune
611 cell infiltration. C, cornea; AC, anterior chamber; L, lens; VC, vitreous chamber; R,
612 retina. **(C)** Scotopic ERG response as measured by the percentage of a-and b-wave
613 amplitudes retained in comparison to uninfected control eyes with values maintained at
614 100%. **(D)** Quantitation of intraocular bacterial burden by serial dilution and plate count
615 represented as CFUs/eye (n=6). **(E)** Corneal opacity was measured using ImageJ and
616 is represented as the integrated pixel intensity. The data represent the culmination of
617 two independent experiments and are shown as the mean \pm SD. Statistical analysis was
618 performed using one-way ANOVA with Tukey's multiple comparisons test (panels C, D,
619 & E). ns = not significant; * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

620 **Fig. 2: Myeloid cell-specific AMPK α 1 deletion enhances inflammation in *S.***

621 ***aureus*-infected eyes. (A-B)** Endophthalmitis was induced in AMPK α 1^{fl} (WT mice) or

622 LysM^{Cre}; AMPK α 1^{fl} (n = 6 per time point: 24, 48, and 72 h) by intravitreal inoculation of
623 *S. aureus* RN6390 (5,00 CFUs/eye). Eyes injected with PBS were used as controls. At
624 the indicated time points, the eyes or retinas were harvested, and ELISA or qPCR was
625 performed to measure the levels of the indicated inflammatory cytokines or chemokines
626 in whole eye lysates or retinas (n = 6). The data represent the culmination of two
627 independent experiments and are shown as the mean \pm SD. Statistical analysis was
628 performed using one-way ANOVA with Tukey's multiple comparisons test (panels A &
629 B). ns = not significant; * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

630 **Fig. 3: *S. aureus*-infected BMDMs lacking AMPK α 1 exhibit a distinct**
631 **transcriptomic signature profile and attenuated phagocytosis.** BMDMs from WT or
632 AMPK α 1 knock-out (KO) mice were infected with *S. aureus* (MOI 10:1) for 6 h
633 (n=3/condition). RNA was extracted, and RNA sequencing was performed using the
634 Illumina platform. **(A)** Volcano plot showing the overall distribution of differentially
635 expressed genes (DEGs). The points represent genes, blue dots indicate no significant
636 difference in genes, red dots indicate upregulated differentially expressed genes, and
637 green dots indicate downregulated differentially expressed genes. The Y-axis shows the
638 adjusted p-value (\log_{10}) and the X-axis shows the \log_2 fold change. **(B)** Gene Ontology
639 enrichment analysis shows the significantly enriched downregulated pathways in KO
640 BMDMs compared to the Wt BMDMs. **(C)** Bubble plot showing enrichment scores for
641 downregulated pathways in KO BMDMs. The size of the sphere is based on the positive
642 enrichment score. **(D)** Heatmap showing DEGs in *S. aureus* (SA)-infected KO vs WT
643 BMDMs.

644 **Fig. 4: AMPK α 1 deficiency promotes M1 macrophage phenotypes in *S. aureus*-**
645 **infected eyes and BMDMs. (A-B)** Endophthalmitis was induced in AMPK α 1^{fl} or
646 LysM^{Cre}; AMPK α 1^{fl} (n = 6 per time point: 24, 48, and 72 h) by intravitreal inoculation of
647 *S. aureus* RN6390 (500 CFUs/eye). Eyes injected with PBS were used as controls. At
648 the indicated time points, the retinas were harvested, and qPCR was performed to
649 measure the expression profiles of M1 and M2 macrophage phenotype markers (n=6).
650 **(C-D)** BMDMs from AMPK α 1^{fl} (WT mice) or LysM^{Cre}; AMPK α 1^{fl} (AMPK α 1 deficient)
651 mice were infected with *S. aureus* (MOI 10:1) for immunostaining (3 h). Immunostaining
652 was performed for iNOS (red) or Arg1 (Green) expression and images were captured at
653 the original magnification of 60X. **(E-F & G-H)** BMDMs from AMPK α 1^{fl} (WT mice) or
654 LysM^{Cre}; AMPK α 1^{fl} mice were infected with *S. aureus* (MOI 10:1) for western blotting (3
655 h). Western blot was performed to detect iNOS, Arg1, and HSP90 proteins.
656 Densitometry analysis was performed using ImageJ and data are expressed as relative
657 fold changes normalized to the loading control, HSP90. The data are representative of
658 two independent experiments and are shown as the mean \pm SD. Statistical analysis was
659 performed using one-way ANOVA with Tukey's multiple comparisons test (panels A &
660 B, E & G). ns = not significant; *P < 0.01; **P < 0.001; ***P < 0.0001.

661 **Fig. 5: Adoptive transfer of wild-type myeloid cells to AMPK α 1 deficient mice**
662 **rescues *S. aureus*-induced endophthalmitis. (A)** Schematic of experimental design
663 for bone marrow transplant and endophthalmitis. **(B-C)** Endophthalmitis was induced in
664 AMPK α 1 KO (recipient), and B6.SJL-*Ptprc*^a *Pepc*^b/BoyJ (PepBoy, donor) (B6
665 background, n = 4, time point: 72 h) by intravitreal inoculation of *S. aureus* RN6390
666 (5,00 CFUs/eye). Eyes injected with PBS were used as controls. Representative slit-

667 lamp micrograph showing corneal haze/opacity. **(C)** Scotopic ERG response as
668 measured by the percentage of a-and b-wave amplitudes retained in comparison to
669 uninfected control eyes with values kept at 100%. **(D)** Corneal opacity was measured using
670 ImageJ and represented as integrated pixel intensity. **(E)** Quantitation of intraocular bacterial
671 burden by serial dilution and plate count represented as CFUs/eye (n=4). **(F & G)** At the
672 indicated time points, the eyes or retinas were harvested and ELISA or flow cytometry
673 was performed to measure the levels of inflammatory cytokines or chemokines from
674 whole eye lysates or neutrophil infiltration from the retinas (n=4). **(H)** Bar graph
675 showing the frequency of neutrophil infiltration in chimeric mice. The data represented
676 are the culmination of two independent experiments and are shown as the mean \pm SD.
677 Statistical analysis was performed using one-way ANOVA with Tukey's multiple
678 comparisons (panels B, & E) or unpaired t-test (C & D). ns = not significant; * $P < 0.01$;
679 ** $P < 0.001$; *** $P < 0.0001$.

680 **Fig. 6. Effect of myeloid cell-specific AMPK α 1 deletion on *S. aureus*-induced**
681 **endophthalmitis.** Mechanistically, myeloid-specific AMPK α 1 inactivation impairs the
682 phagocytic clearance of *S. aureus* and the resolution of inflammation by skewing toward
683 the M1 macrophage phenotype. The adoptive transfer of AMPK-competent bone
684 marrow from WT mice to AMPK α 1 deficient mice preserved retinal functions and
685 attenuated disease severity.

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694 **Supplementary Figures.**

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697 **Fig. S1: Myeloid cell-specific AMPK α 1 loss enhances the inflammation in *S.***
698 ***aureus*-infected BMDMs.** BMDMs from AMPK α 1^{fl} or LysM^{Cre}; AMPK α 1^{fl} (n=4/condition)
699 were infected with *S. aureus* (MOI 10:1) for 6h. The expression of inflammatory
700 cytokines was measured by **(A)** qPCR and **(B)** ELISA. The data represent the
701 culmination of two independent experiments and are shown as the mean \pm SD.
702 Statistical analysis was performed using one-way ANOVA with Tukey's multiple
703 comparisons test (panels A & B). ns = not significant; * $P < 0.01$; ** $P < 0.001$; *** $P <$
704 0.0001 .

705 **Fig. S2: AMPK α deficient BMDMs exhibit attenuation of antimicrobial gene**
706 **expression and phagocytosis.** **(A)** BMDMs from AMPK α 1^{fl} or LysM^{Cre}; AMPK α 1^{fl}
707 (n=4/condition) were infected with *S. aureus* (MOI 10:1) for 6 h. The expression profiles
708 of genes (e.g., *S100a7*, *S100a8*, and *S100a10*) with antimicrobial functions were
709 assessed by qPCR. **(B)** BMDMs from AMPK α 1^{fl} or LysM^{Cre}; AMPK α 1^{fl} (n=4/condition)
710 were challenged with *S. aureus* (MOI 10:1) for 2 h. After 2 h of infection, the cells were
711 rinsed to remove extracellular bacteria and incubated with fresh medium containing

712 gentamicin (200 µg/mL) for the indicated time points. At the desired time point, the cells
713 were lysed, and the viable bacterial counts were quantitated via serial dilution and
714 counting. Statistical analysis was performed using one-way ANOVA with Tukey's
715 multiple comparison test (panels A, & B). The data represents mean ± SD from three
716 independent experiments. ns = not significant; ** $P < 0.01$; *** $P < 0.001$.

Fig 1

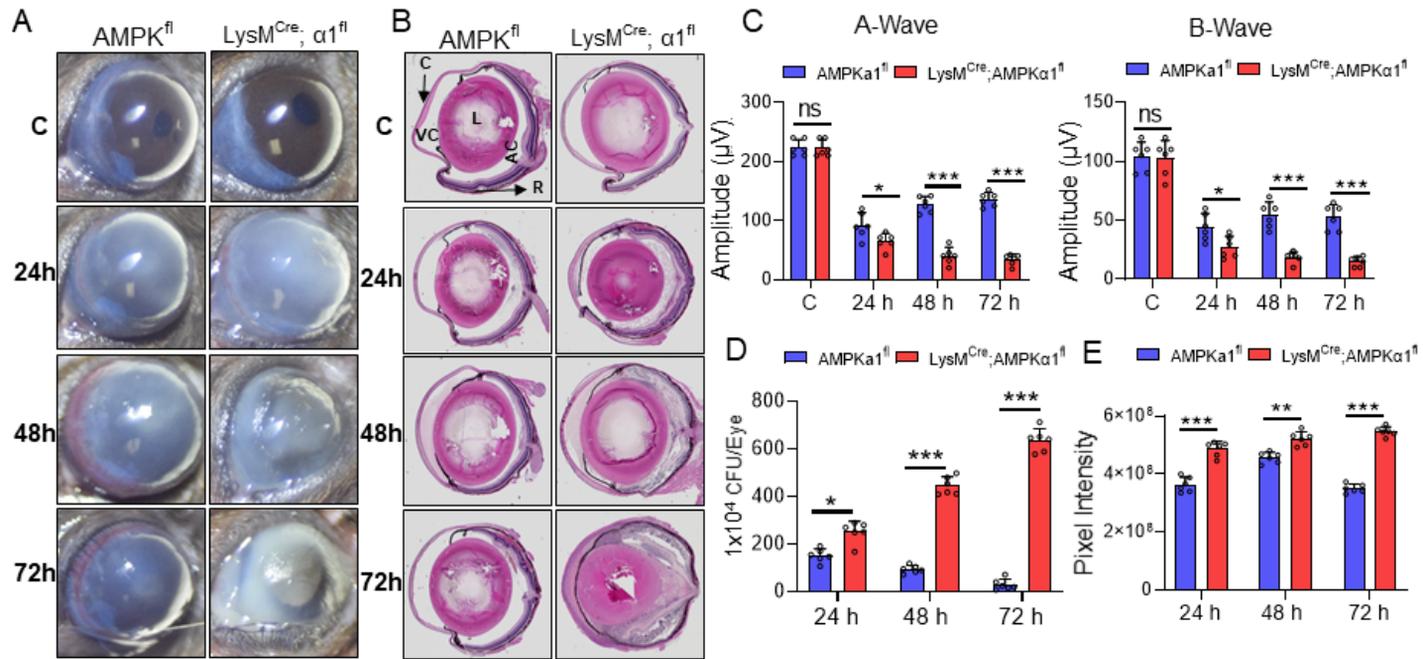


Fig 2

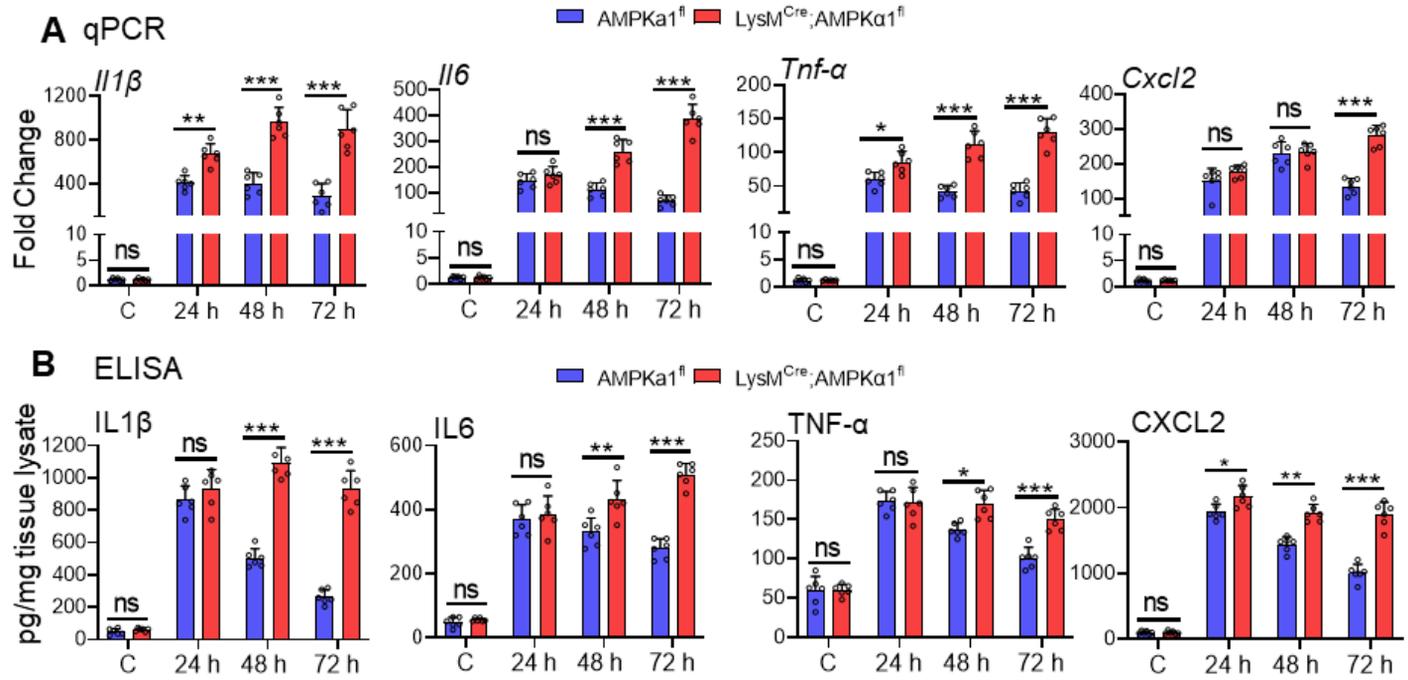


Fig 3

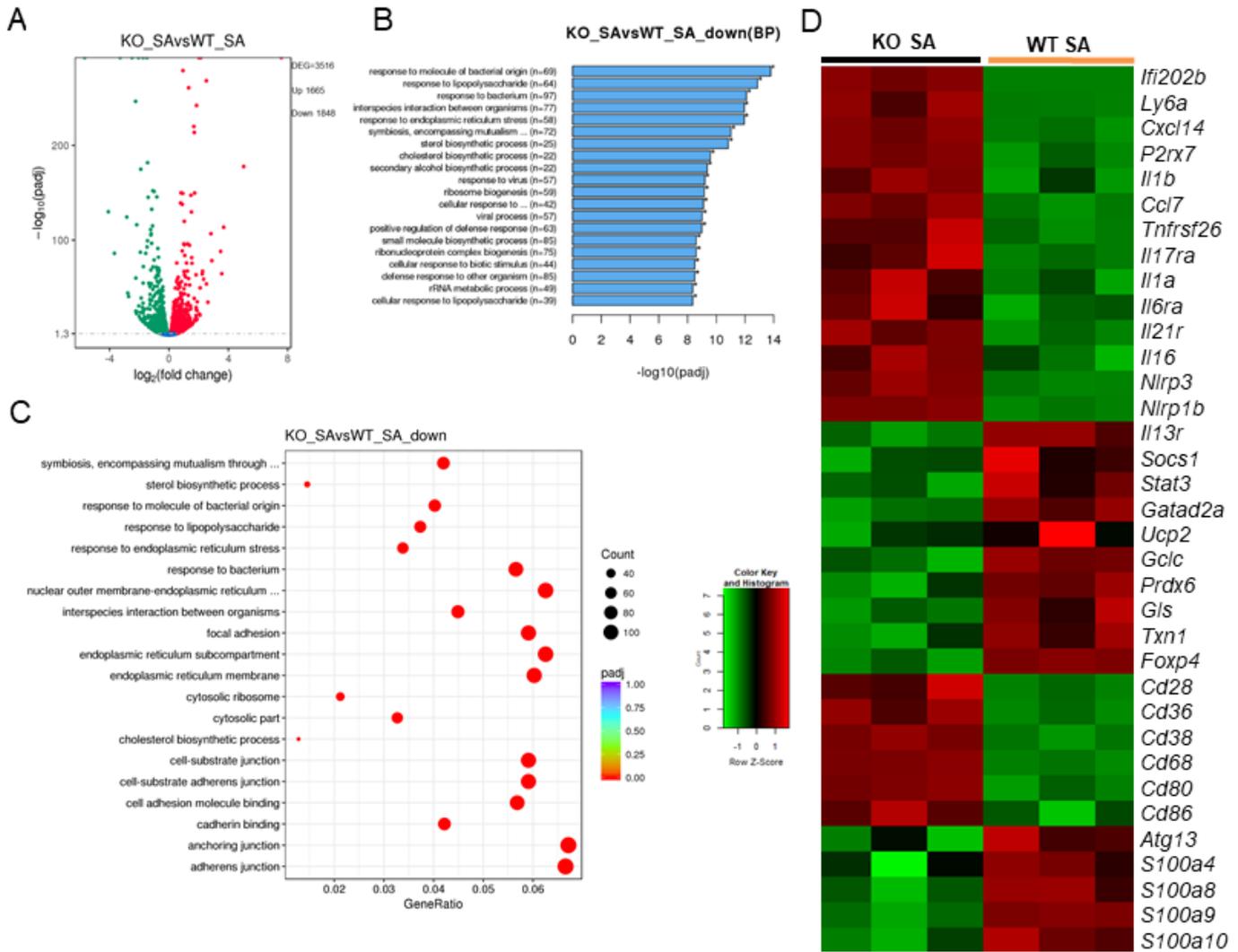
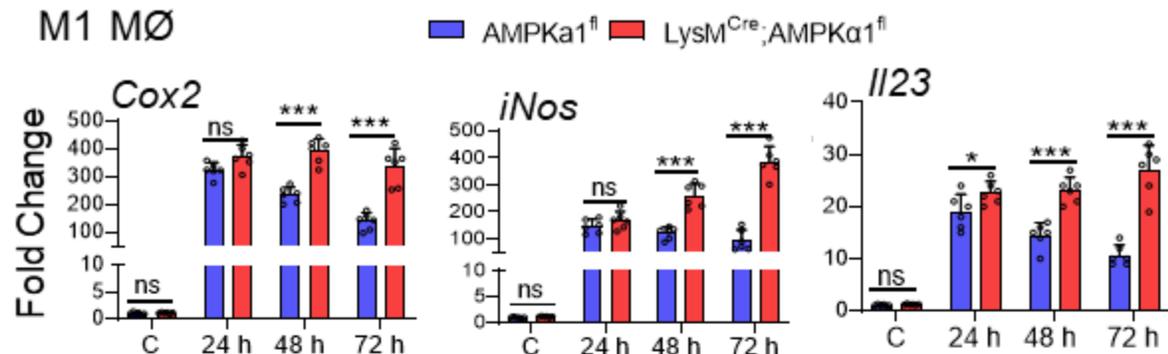
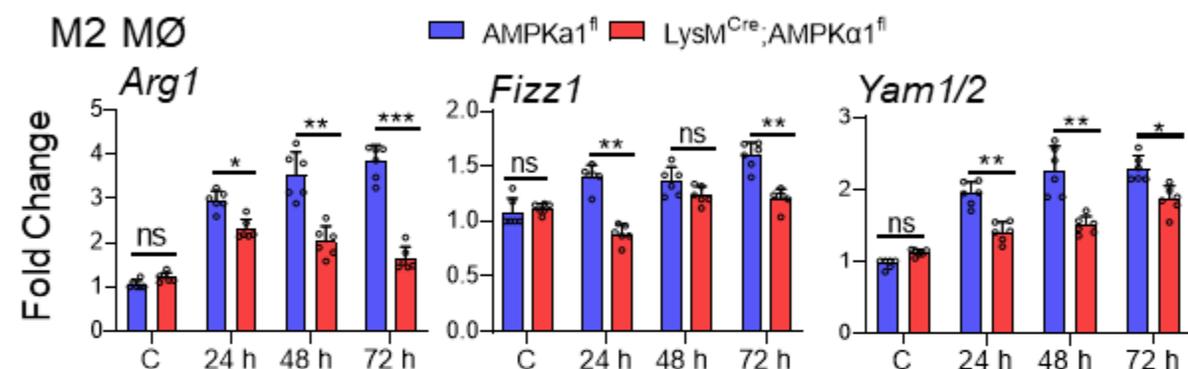


Fig 4

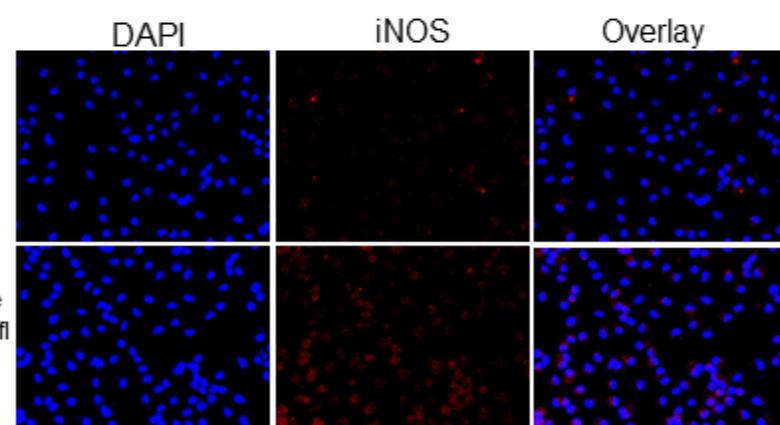
A M1 MØ



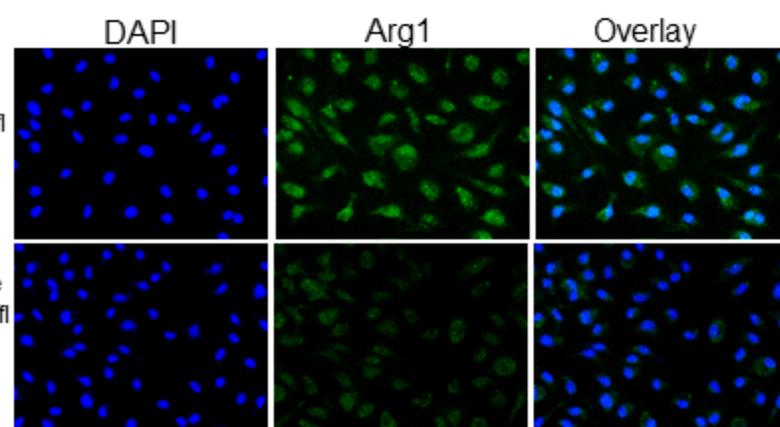
B M2 MØ



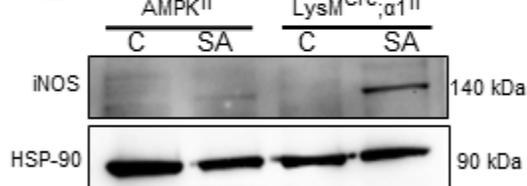
C



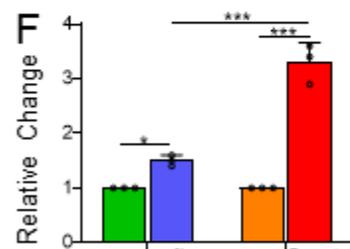
D



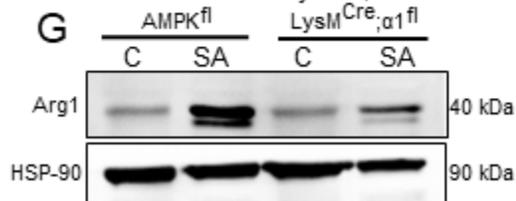
E



F



G



H

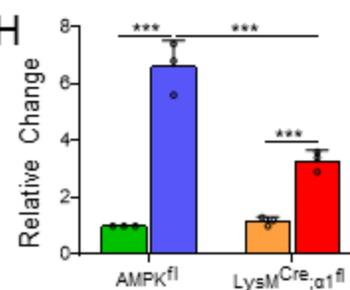


Fig 5

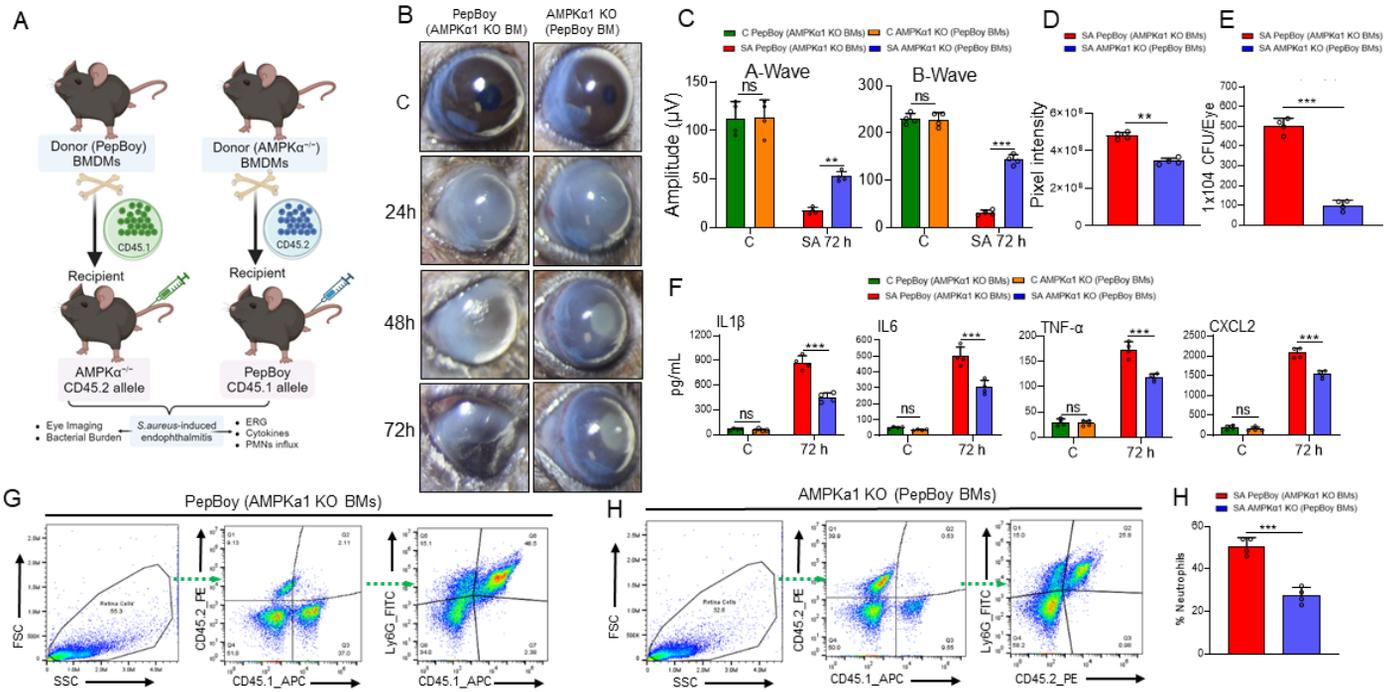


Fig 6

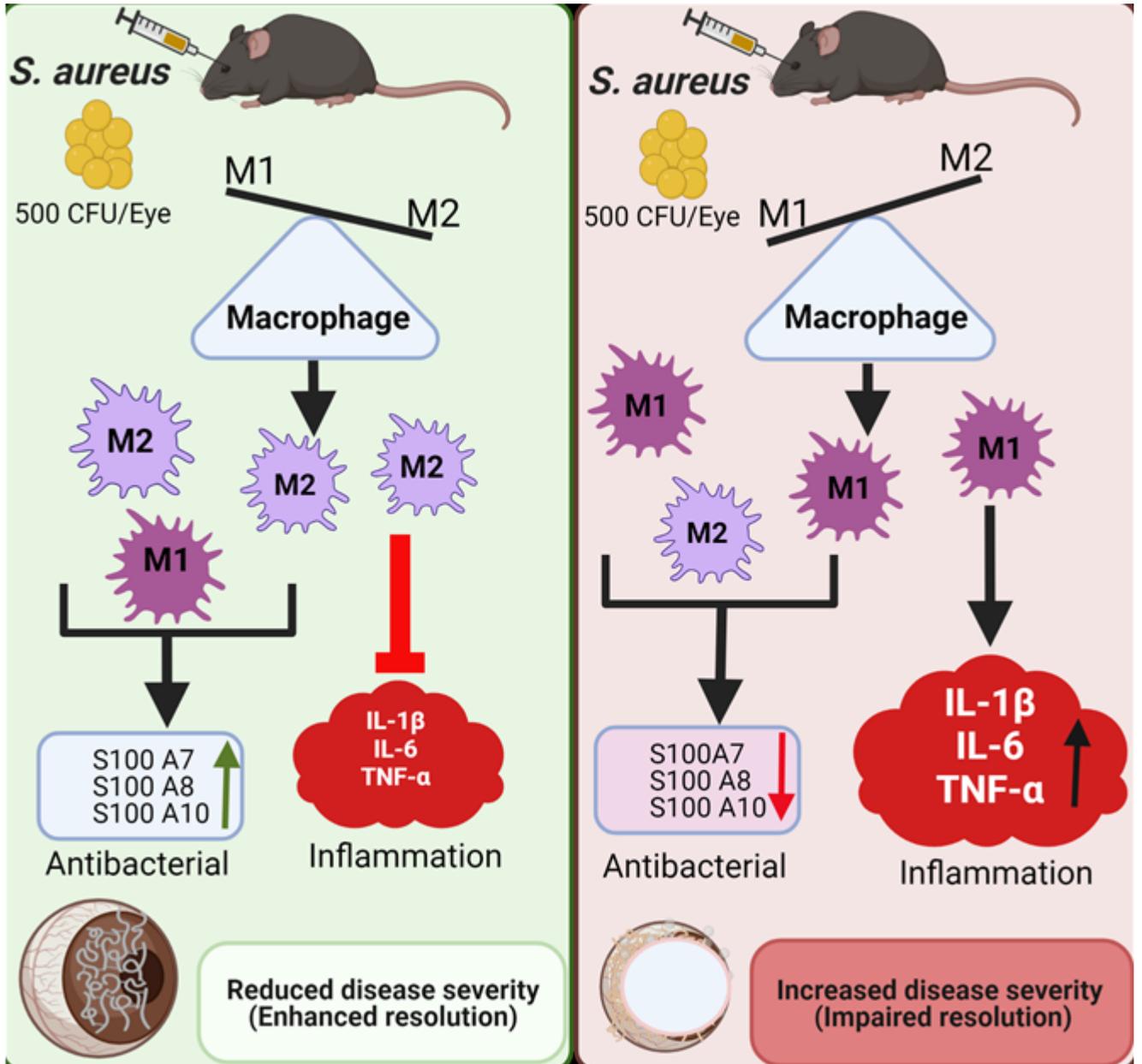


Fig 1S

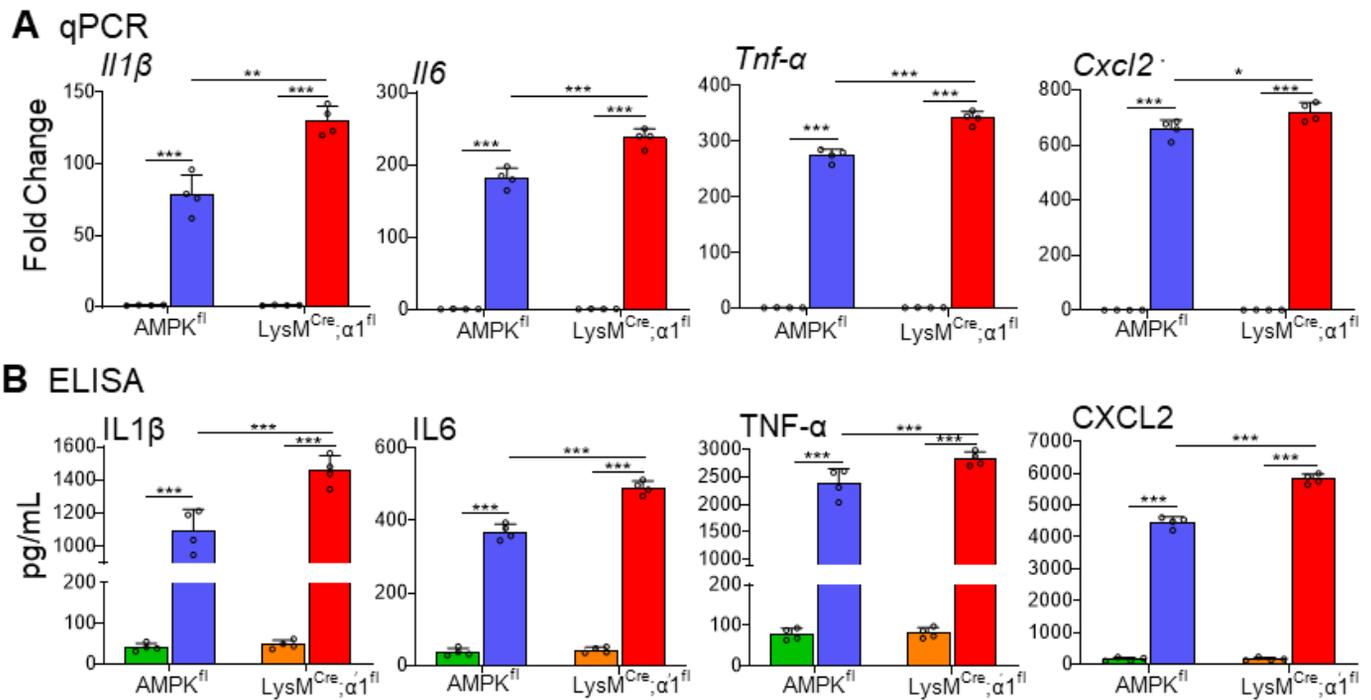


Fig 2S

