KCC1 Activation protects Mice from the Development of Experimental Cerebral Malaria.

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Abstract

Plasmodium falciparum malaria causes half a million deaths per year, with up to 9% of this mortality caused by cerebral malaria (CM). One of the major processes contributing to the development of CM is an excess of host inflammatory cytokines. Recently K+ signaling has emerged as an important mediator of the inflammatory response to infection; we therefore investigated whether mice carrying an ENU-induced activation of the electroneutral K+ channel KCC1 had an altered response to *Plasmodium berghei*. Here we show that *Kcc1*$^{M935K/M935K}$ mice are protected from the development of experimental cerebral malaria, and that this protection is associated with an attenuated INF-ɣ response. This is the first description of a K+ channel affecting the development of cerebral malaria.

Introduction

Plasmodium falciparum malaria is a major cause of mortality worldwide, leading to an estimated 429,000 deaths per year[1]. One of the most severe and lethal complications of *P. falciparum* infection is the sudden onset of seizures and/or coma known as cerebral malaria (CM). Its occurrence varies from region to region, with a case fatality rate as high as 9% of severe malaria cases in some areas [2,3]. The causes of CM are not well understood, but hypotheses include both the accumulation of parasitized red blood cells in the brain microvasculature, as well as imbalance in the pro- and anti- inflammatory responses to infection[4].

In recent years, potassium (K+) signaling has emerged as an important mediator of the immune response to infection. Several studies have shown *in vitro* that functional outwardly rectifying K+ channels are necessary for macrophage activation and production of TNFα[5,6], for activation of the NALP inflammasome[7], for the activation of T helper cells, and the formation of T regulatory cells [8-10]. The K+ content of the RBC also has a large effect on intra-erythrocytic *Plasmodium*. It has been shown that an outwardly directed K+ gradient is needed for normal parasite growth and maintenance of the parasite plasma membrane potential [11-13].

A mouse line expressing an activated form of KCC1, discovered from a large scale ENU mutagenesis screen, has recently been described[14]. The induced mutation – an M to K substitution at amino acid 935 of the protein – impairs phosphorylation of neighboring regulatory threonines, leading to aberrant activity of the transporter. The resulting increase in K+ efflux from the RBC causes *Kcc1*^{M935K} mice to display a microcytic anemia, with homozygous mutants showing a 21% decrease in Mean corpuscular Volume (MCV), 8% decrease in total hemoglobin, and 21% increase in number of red cells. Mutant cells are also significantly less osmotically fragile[14] indicating a dehydration of the red blood cells.

Here we use the *Kcc1*^{M935K} mice line to investigate the effect of increased host K+ efflux on susceptibility to malaria infection. When *Kcc1*^{M935K} mice were infected with *Plasmodium berghei,* they showed a marked protection from the development of experimental cerebral malaria (ECM), associated with a significant decrease in IFNɣ and IL-10 during infection, suggesting K+ efflux through KCC1 attenuates the inflammatory response to infection. This is the first description of a cation transporter affecting the development of ECM.

Results

Kcc1^{M935K} *has increased survival to P. berghei challenge*

Kcc1$^{M935K/M935K}$ mice were inoculated with *P. berghei* to determine their resistance to infection. Cumulative survival and peripheral parasitemia were monitored daily over the course of infection. When mice were infected with $1x10^4$ *P. berghei* parasitized red cells, survival was significantly increased in the mutants, with 100% of homozygotes surviving past day 10 of infection, compared to 7% of WT females (P=0.0004; Figure 1A), and 11% of WT males ($P=2.66x10^{-7}$; Figure1B). Significantly lower parasitemia was observed in both *Kcc1*^{M935K} females and males. In females, parasitemia was reduced by 63% on day

91 7, 48% on day 8, and 42% on day 9 post inoculation. Parasitemia in males was
92 similarly reduced, by 66%, 41%, and 53% respectively (Figure 1A and B). To
93 determine if this reduction in parasitemia was caused by impaired parasitic
94 invasion of *Kcc1*$^{M935K/M935K}$ RBCs and survive within them, we conducted TUNEL
95 staining of infected RBCs to detect fragmented nuclei in the parasites indicative
96 of maturation arrest, and an *in vivo* invasion assay as previously described[15]. No
97 significant differences were observed in either assay (Figure 1C and D),
98 suggesting the *Kcc1*^{M935K} mutation does not affect parasite invasion or growth.
99

100 Kcc1^{M935K} *is resistant to ECM*

101 When infected with *P. berghei* in the experiments described above, most WT
102 mice died 8 to 10 days after infection. To determine if the WT mice dying from *P.*
103 *berghei* infection were succumbing to experimental cerebral malaria (ECM), mice
104 were injected with *P. berghei* and symptoms of ECM were scored according to
105 severity, from 0 (no symptoms) to 5 (death). Severe clinical symptoms were
106 observed in WT mice, with most dying from seizures, whereas Kcc1$^{M935K/M935K}$
107 remained asymptomatic for the length of the experiment (Figure 2B). One of the
108 key hallmarks of cerebral malaria is breakdown of the blood brain barrier.
109 Therefore, mice were injected intravenously with Evan's Blue to assess blood
110 brain barrier integrity. Infected WT mice showed an average of 14.5±2.9 grams
111 of dye per gram of brain tissue, which was significantly higher than the
112 8.1±1.4g/g observed in non-infected mice. Infected Kcc1$^{M935K/M935K}$ did not differ
113 significantly from non-infected mice with 9.4 ± 1.3 g/g (Figure 2A and C). These
114 results indicate a significant breakdown of the blood brain barrier in WT mice
115 that is not observed in the mutants. Together with the clinical scores, this
116 suggests that Kcc1$^{M935K/M935K}$ mice are resistant to the development of ECM.
117

118 Kcc1^{M935K} *has an abnormal immune response to infection*

119

120 It has been shown that depletion of CD4+ T cells, CD8+ T cells, and inflammatory
121 monocytes can prevent the development of ECM [16-18]. ECM resistance is also
122 observed in mice with impaired thymic development of CD8+ T cells[19]. Because

123 KCC1 is expressed ubiquitously[20], it was hypothesized that the *Kcc1*^{M935K}
124 mutation might cause alterations to some of these immune cell populations.
125 Therefore, the relative proportion of CD3+, CD11c+, and CD19+ cells were
126 measured by flow cytometry in the blood, spleen and thymus, both in non-
127 infected mice, and day 8 of *P. berghei* infection where the inflammatory response
128 is expected to be highest[21,22]. No significant differences in these cell populations
129 were observed in any of these tissues, before or during infection (Figure 3).
130 CD3+ cells were further analyzed to determine the relative proportion of CD4+
131 and CD8+ subsets. Again, no significant differences were observed. In WT mice
132 the average proportion of CD4+ cells rose with infection in the blood, spleen, and
133 thymus, from 41 to 62%, 37 to 75%, and 40 to 56% respectively. This trend was
134 also observed in *KCC1*$^{M935K/M935K}$ where CD4+ cells rose from an average
135 proportion of 36 to 62%, 34 to 78%, and 35 to 48% respectively (Figure 3). The
136 average proportion of CD8+ also increased in the blood with infection, from 22 to
137 32% in WT mice, and from 28 to 31% in *KCC1*$^{M935K/M935K}$. Conversely, in both the
138 spleen and thymus the average proportion of CD8+ cells was similar between
139 uninfected and infected animals. Together we noted no difference in T-cell
140 response.
141
142 One of the major host processes known to contribute to the development of
143 cerebral malaria in *P. berghei* infection is an over-active cytokine-driven
144 inflammatory response. Both *in vivo* neutralisation of host molecules, and studies
145 with knock-out mice have shown that cerebral malaria can be prevented by
146 depletion of the pro-inflammatory cytokines IFN-γ [23,24] and TNFα [25], and can be
147 induced by depletion of the anti-inflammatory cytokine IL-10 [26]. CM resistance is
148 also observed in mice with defective T cell dependent IFN-γ production [19]. We
149 therefore measured plasma cytokine levels in infected mice over the course of
150 *P. berghei* infection.
151
152 As expected, WT mice showed strong increases in both IFN-γ and TNFα over the
153 course of infection, with IFN-γ peaking around the onset of symptoms at day 9.
154 IL-10 was variable over the course of infection, and IL-6 showed a smaller, late
155 stage response. *Kcc1*^{M935K} mice displayed similar trends, but differed significantly

156 from WT in several key points. On day 7 post infection, *Kcc1*^{M935K} showed a 1.2-
157 fold increase in TNFα, which was followed on day 9 by a 0.6-fold reduction in the
158 amount of IFN-γ, and a 0.8-fold reduction in the amount of IL-6 compared to WT.
159 On day 10 of infection *Kcc1*^{M935K} displayed a 0.9-fold reduction in the amount of
160 IL-10 compared to WT (Figure 4).
161

162 **Discussion**

163

164 This study provides the first evidence that host KCC1 plays a role in malaria
165 resistance. It shows that over-activation of the transporter causes resistance to
166 experimental cerebral malaria (ECM) in *P. berghei* infection. This is the first
167 description of a mutation in a cation transporter that has an effect on ECM; other
168 previously discovered genes have involved host cytokines, antigen
169 presentation [27,28], or erythrocyte membrane proteins [29-31].
170

171 Whereas the vast majority of non-mutant littermates succumbed to a classical
172 ECM, all homozygous mutant animals survived the infection and did not develop
173 any symptoms of the syndrome. Resistance in the *Kcc1*$^{M935K/M935K}$ animals was
174 accompanied with a significantly lower parasitemia than WT during the first 10
175 days of infection. However the mutation did not appear to have a red cell
176 autonomous effect on either parasite invasion or survival within the RBC. A
177 possible but untested explanation for the reduced parasitemia effect is the
178 mutation affects the cytoadherence of infected red cells to the endothelium of
179 blood vessels. Lower levels of sequestration in the mutants would leave more
180 late stage parasites vulnerable to splenic clearance, and therefore result in
181 reduced parasite burden[32]. Reduced sequestration would also be consistent with
182 protection from cerebral malaria, as infected cells would be less likely to adhere
183 within the microvasculature of the brain.

184 All of the T cell populations examined in the spleen, thymus and circulation were
185 similar between mutant and WT mice, and none changed appreciably during the
186 *Plasmodium berghei* infection. However, production of several cytokines
187 previously shown to direct the ECM response [23-25] were shown to be attenuated

188 in the mutant animals, particularly IFN-γ and IL10. It is not clear from this study
189 exactly what is causing decreased INF-γ and IL-10 in *Kcc1*^{M935K}. This reduced
190 inflammation may simply be the result of the lower parasitemia observed in
191 mutants. However, KCC1 is expressed on a wide range of immune cells, and this
192 may affect their function. Previous studies have shown that K+ efflux can alter
193 cellular cytokine production[5-7]; can increase assembly of the NALP
194 inflammasome in response to pathogen associated proteins[7]; and is essential
195 macrophage migration[33]. All of these may help contribute to both the attenuated
196 inflammatory response, and the lower parasitemia observed in *Kcc1*^{M935K} mice.
197
198 Here we have shown that activation of KCC1 causes a dramatic resistance to
199 *P. berghei* by completely preventing the development of experimental cerebral
200 malaria (ECM). This is the first description of a mutation in a transporter that has
201 an effect on ECM. Previous studies have shown that pharmacological activation
202 of KCC channels is achiavable[34,35], therefore future research into KCC1 activation
203 may provide novel treatments for cerebral malaria.
204

205 ## Methods

206 ### Animals

207 Mice were bred under specific pathogens free conditions. All procedures
208 conformed to the National Health and Medical Research Council (NHMRC) code
209 of practice. All mouse procedures have been approved by the Australian National
210 University Animal Experimentation Ethics Committee (AEEC A2014/054). The
211 Kcc1^{M935K} mutation is carried on a mixed BALB/c and C57BL/6 background[14].
212 These two mouse strains differ in their susceptibility to *P. berghei*, and this
213 introduced a greater amount of variability into results than is usually observed.
214 Therefore, WT x WT and Kcc1$^{M935K/M935K}$ x Kcc1$^{M935K/M935K}$ breeding pairs were
215 maintained. To exclude the possibility that the resistance phenotype was due to
216 the mixed background, and carried by chance in mutant breeding pairs,
217 Kcc1^{M935K} was periodically crossed back to WT, and new WT x WT and
218 Kcc1$^{M935K/M935K}$ x Kcc1$^{M935K/M935K}$ pairs established from the progeny.
219

220 ### Infections

221 Experiments used either the rodent parasite *P. berghei ANKA*. Parasite stocks
222 were prepared from passage through resistant SJL/J mice, as described
223 previously[36]. Experimental mice were infected intraperitoneally at a dose of
224 $1x10^4$ parasitised RBC. Blood stage parasitemia was determined by counting thin
225 smears from tail blood stained in 10% Giemsa solution. A least 300 cells were
226 counted per slide.
227
228 **Histology**
229 Thin tail smears from *P. berghei* infected mice were fixed in 100% MeOH, and
230 stained with an APO-BrdU TUNEL assay kit according to the manufacturer's
231 instructions (Invitrogen, Carlsbad, CA). Slides were examined on an upright
232 epifluorescence microscope (ZIESS) 600x magnification. 10 fields of view were
233 counted for each slide.
234
235 **Evans Blue**
236 *P. berghei* infected mice, and uninfected controls, were injected IV with 200µl 1%
237 Evans Blue/PBS solution. 1hr post injection, mice were sacrificed and their
238 brains collected and weighed. Brains were placed in 2ml 10% neutral buffered
239 formalin at room temperature for 48hrs to extract dye. 200µl of formalin from
240 each brain was then collected and absorbance measured at 620nm. Amount of
241 Evans blue extracted per gram brain tissue was calculated using a standard
242 curve ranging from 40µg/ml to 0µg/ml. Injections were carried out on the day of
243 infection that the first mouse died.
244
245 **Clinical Score**
246 Mice were monitored three times daily, and given a score from 0 to 5 based on
247 the type and severity of their symptoms. '0' indicated no symptoms; '1' reduced
248 or languid movement; '2' rapid breathing and/or hunched posture; '3' ruffled fur,
249 dehydration and/or external bleeding; '4' fitting and/or coma; '5' death. Mice
250 were considered comatose if they were unable to right themselves after being
251 placed on their side. The highest score recorded for each mouse on each day was
252 used to generate daily averages.
253

254 **Cytokines**

255 Peripheral blood was taken either by cardiac puncture or mandibular bleed and
256 centrifuged for 4 minutes at 11,000xg. Plasma was then taken into a separate tube and
257 stored at -20°C until needed. Cytokine analysis was conducted on un-diluted plasma
258 using a CBA Mouse Th1/Th2/Th17 Cytokine Kit to the maker's instructions (BD
259 biosciences).

260

261 **Lymphocyte Analysis**

262 Peripheral blood was taken either by cardiac puncture or mandibular bleed, and
263 lymphocytes were isolated on Ficoll-Paque™ according to the maker's instructions.
264 Lymphocytes were then incubated with Fc-block in MT-FACS for 10 minutes at 4°C.

265

266 Both spleen and thymus were prepared for flow cytometry using the same method. ½
267 of each organ was passed through a 70µm BD Falcon® Cell Strainer with 0.5 ml of
268 MT-FACS buffer, and then centrifuged at 300xg for 5 minutes at 4°C. The
269 supernatant was removed and the pellet re-suspended in 5 ml cold MT-FACS buffer.
270 A 200µl aliquot of this suspension was then incubated with 0.8µl Fc-block.

271

272 Blood, spleen and thymus samples were then stained with CD4-PacificBlue, CD11-
273 PE, CD8-FITC, CD25-PECy7, CD19-PERCPCy5.5 and CD3-APC-Cy7, then fixed
274 with 300µl of MT-PBS containing 1% formalin and 1% BSA for 10 minutes at 4°C.
275 Cells were then permeabilised with MT-PBS containing 0.1% saponin and 1%BSA at
276 4°C for 20 minutes. Finally, cells were stained with FoxP3-APC. Samples were
277 acquired using a BD FACSAria™ II flow cytometer, and analysed using BD
278 FACSDiva™ software (BD Biosciences).

279

280 **TUNEL staining**

281 Thin tail smears from *P. chabaudi* infected mice were fixed in 100% MeOH. For all
282 TUNEL staining, an APO-BrdU TUNEL assay kit was used (Invitrogen, Carlsbad,
283 CA). Slides were initially washed three times with 1ml of wash buffer solution before
284 being incubated with 50µl of DNA labelling solution mix (according to
285 manufacturer's instructions) overnight at room temperature. The next day slides were
286 rinsed three times with 1ml rinse solution with two minutes incubation time each.
287 Stained sections were then incubated with 100µl of BrdU-antibody for one hour in the

288 dark at room temperature with subsequent washes in 1%BSA/ 1x MT-PBS (three
289 times with a two minute soak each). Slides were then further labelled with 100µl of
290 nuclear yellow (Invitrogen, Carlsbad, CA) (1:5000 in 1% BSA/ 1 x MT-PBS) for one
291 minute in the dark. After further washes with 1ml MT-PBS (three times with a two
292 minute soak each) sections were affixed with Fluorescent Mounting Medium
293 (DakoCytomation). Once the medium dried, slides were examined on an upright
294 epifluorescence microscope (ZIESS) 600x magnification. 10 fields of view were
295 counted for each slide.

296

297 ***In-vivo* Invasion Assay**

298 Blood from Mutant and WT uninfected mice was collected by cardiac puncture.
299 1800µl of blood was collected and pooled for each genotype, then halved and stained
300 with either NHS-Atto 633 (1µl/100µl) or sulfobiotin-LC-NHS-Biotin (1µl/100µl of
301 25mg/ml in DMF). Cells were then incubated at RT for 30 minutes, and washed twice
302 in MTPBS. Stained cells were combined in equal proportions to achieve the following
303 combinations:

304 1) WT-Biotin + Mutant-Atto 2) WT-Atto + Mutant Biotin

305 Combined cells were then resuspended in 2ml MTPBS, and injected intravenously
306 into WT *P. berghei* infected mice. 4 mice at 1-5% parasitemia, plus 1 uninfected
307 control, were injected with 200µl dye combination 1; the same numbers of mice were
308 injected with 200µl dye combination 2. Injections were carried out when parasites
309 were undergoing schizogeny, at ~1am.

310 30 minutes post injection, 1µl tail blood was collected and stained for 30 minutes at
311 4°C in 50µl MTPBS containing 0.25µl CD45-APC-Cy7, 0.25µl CD71-PE-Cy5, 0.5µl
312 Step-PE-Cy7. Next, 400µl MTPBS containing 0.5µl Hoechst 33342 and 1µl
313 800µg/ml Thiazole orange was added, and cells were incubated for a further 5
314 minutes at 4°C. Stained cells were then centrifuged at 750xg for 3 minutes, re-
315 suspended in 700µl MTPBS, and analyzed on a BD Fortessa Flow Cytometer. $2x10^6$
316 cells were collected for each sample, and data was analysed using FlowJo (FlowJo,
317 LLC, Oregon, USA).

318

319

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322 **References**

323

324 1 WHO. World Malaria Report. 198 (World Health Organization, 2016).
325 2 Grau, G. E. & Craig, A. G. Cerebral malaria pathogenesis: revisiting parasite
326 and host contributions. *Future microbiology* **7**, 291-302, (2012).
327 3 Rockett, K. A. *et al.* Reappraisal of known malaria resistance loci in a large
328 multicenter study. *Nat Genet* **46**, 1197-1204, (2014).
329 4 Brooks, H. M. & Hawkes, M. T. Repurposing Pharmaceuticals as
330 Neuroprotective Agents for Cerebral Malaria. *Curr Clin Pharmacol*, (2017).
331 5 Qiu, M. R., Campbell, T. J. & Breit, S. N. A potassium ion channel is involved
332 in cytokine production by activated human macrophages. *Clin Exp*
333 *Immunol* **130**, 67-74 (2002).
334 6 Ren, J. D. *et al.* Involvement of a membrane potassium channel in heparan
335 sulphate-induced activation of macrophages. *Immunology* **141**, 345-352,
336 (2014).
337 7 Petrilli, V. *et al.* Activation of the NALP3 inflammasome is triggered by low
338 intracellular potassium concentration. *Cell Death Differ* **14**, 1583-1589,
339 (2007).
340 8 Bittner, S. *et al.* Upregulation of K2P5.1 potassium channels in multiple
341 sclerosis. *Ann Neurol* **68**, 58-69, doi:10.1002/ana.22010 (2010).
342 9 Fellerhoff-Losch, B. *et al.* Normal human CD4(+) helper T cells express
343 Kv1.1 voltage-gated K(+) channels, and selective Kv1.1 block in T cells
344 induces by itself robust TNFalpha production and secretion and
345 activation of the NFkappaB non-canonical pathway. *J Neural Transm*
346 *(Vienna)* **123**, 137-157, (2016).
347 10 Wulff, H. *et al.* The voltage-gated Kv1.3 K(+) channel in effector memory T
348 cells as new target for MS. *J Clin Invest* **111**, 1703-1713, (2003).
349 11 Staines, H. M., Ellory, J. C. & Kirk, K. Perturbation of the pump-leak balance
350 for Na(+) and K(+) in malaria-infected erythrocytes. *Am J Physiol Cell*
351 *Physiol* **280**, C1576-1587 (2001).
352 12 Brand, V. B. *et al.* Dependence of Plasmodium falciparum in vitro growth
353 on the cation permeability of the human host erythrocyte. *Cellular*
354 *physiology and biochemistry : international journal of experimental cellular*
355 *physiology, biochemistry, and pharmacology* **13**, 347-356, (2003).
356 13 Allen, R. J. & Kirk, K. The membrane potential of the intraerythrocytic
357 malaria parasite Plasmodium falciparum. *J Biol Chem* **279**, 11264-11272,
358 (2004).
359 14 Brown, F. C. *et al.* Activation of the erythroid K-Cl cotransporter Kcc1
360 enhances sickle cell disease pathology in a humanized mouse model.
361 *Blood* **126**, 2863-2870, (2015).
362 15 Lelliott, P. M., McMorran, B. J., Foote, S. J. & Burgio, G. In vivo assessment
363 of rodent Plasmodium parasitemia and merozoite invasion by flow
364 cytometry. *J Vis Exp*, e52736, (2015).
365 16 Claser, C. *et al.* CD8+ T cells and IFN-gamma mediate the time-dependent
366 accumulation of infected red blood cells in deep organs during
367 experimental cerebral malaria. *PLoS One* **6**, e18720, (2011).
368 17 Hermsen, C., van de Wiel, T., Mommers, E., Sauerwein, R. & Eling, W.
369 Depletion of CD4+ or CD8+ T-cells prevents Plasmodium berghei induced

370 cerebral malaria in end-stage disease. *Parasitology* **114 (Pt 1)**, 7-12
371 (1997).
372 18 Schumak, B. *et al.* Specific depletion of Ly6C(hi) inflammatory monocytes
373 prevents immunopathology in experimental cerebral malaria. *PLoS One*
374 **10**, e0124080, (2015).
375 19 Bongfen, S. E. *et al.* An N-Ethyl-N-Nitrosourea (ENU)-Induced Dominant
376 Negative Mutation in the JAK3 Kinase Protects against Cerebral Malaria.
377 *PloS one* **7**, e31012, (2012).
378 20 Gillen, C. M., Brill, S., Payne, J. A. & Forbush, B., 3rd. Molecular cloning and
379 functional expression of the K-Cl cotransporter from rabbit, rat, and
380 human. A new member of the cation-chloride cotransporter family. *J Biol*
381 *Chem* **271**, 16237-16244 (1996).
382 21 Hanum, P. S., Hayano, M. & Kojima, S. Cytokine and chemokine responses
383 in a cerebral malaria-susceptible or -resistant strain of mice to
384 Plasmodium berghei ANKA infection: early chemokine expression in the
385 brain. *Int Immunol* **15**, 633-640 (2003).
386 22 Lacerda-Queiroz, N. *et al.* Inflammatory changes in the central nervous
387 system are associated with behavioral impairment in Plasmodium
388 berghei (strain ANKA)-infected mice. *Exp Parasitol* **125**, 271-278, (2010).
389 23 Yanez, D. M., Manning, D. D., Cooley, A. J., Weidanz, W. P. & van der Heyde,
390 H. C. Participation of lymphocyte subpopulations in the pathogenesis of
391 experimental murine cerebral malaria. *J Immunol* **157**, 1620-1624 (1996).
392 24 Amani, V. *et al.* Involvement of IFN-gamma receptor-medicated signaling
393 in pathology and anti-malarial immunity induced by Plasmodium berghei
394 infection. *Eur J Immunol* **30**, 1646-1655 (2000).
395 25 Grau, G. E. *et al.* Tumor necrosis factor (cachectin) as an essential
396 mediator in murine cerebral malaria. *Science* **237**, 1210-1212 (1987).
397 26 Kossodo, S. *et al.* Interleukin-10 modulates susceptibility in experimental
398 cerebral malaria. *Immunology* **91**, 536-540 (1997).
399 27 Koch, O. *et al.* IFNGR1 gene promoter polymorphisms and susceptibility
400 to cerebral malaria. *J Infect Dis* **185**, 1684-1687, (2002).
401 28 Hill, A. V. *et al.* Common west African HLA antigens are associated with
402 protection from severe malaria. *Nature* **352**, 595-600, (1991).
403 29 Cortes, A., Benet, A., Cooke, B. M., Barnwell, J. W. & Reeder, J. C. Ability of
404 Plasmodium falciparum to invade Southeast Asian ovalocytes varies
405 between parasite lines. *Blood* **104**, 2961-2966, (2004).
406 30 Fischer, P. R. & Boone, P. Short report: severe malaria associated with
407 blood group. *Am J Trop Med Hyg* **58**, 122-123 (1998).
408 31 Fry, A. E. *et al.* Common variation in the ABO glycosyltransferase is
409 associated with susceptibility to severe Plasmodium falciparum malaria.
410 *Hum Mol Genet* **17**, 567-576, (2008).
411 32 Simpson, J. A., Aarons, L., Collins, W. E., Jeffery, G. M. & White, N. J.
412 Population dynamics of untreated Plasmodium falciparum malaria within
413 the adult human host during the expansion phase of the infection.
414 *Parasitology* **124**, 247-263 (2002).
415 33 Kan, X. H. *et al.* Kv1.3 potassium channel mediates macrophage migration
416 in atherosclerosis by regulating ERK activity. *Arch Biochem Biophys* **591**,
417 150-156, (2016).

34 Delpire, E. & Kahle, K. T. The KCC3 cotransporter as a therapeutic target for peripheral neuropathy. *Expert Opin Ther Targets* **21**, 113-116, (2017).
35 Yamada, K. *et al.* Small-molecule WNK inhibition regulates cardiovascular and renal function. *Nat Chem Biol* **12**, 896-898, (2016).
36 Lelliott, P. M., Lampkin, S., McMorran, B. J., Foote, S. J. & Burgio, G. A flow cytometric assay to quantify invasion of red blood cells by rodent Plasmodium parasites in vivo. *Malar J* **13**, 100, (2014).

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Author Contribution Statement:

E.J.H, B.J.M, S.F.J and G.B designed and planed the experimental work. E.J.H, and F.C.B performed the research. E.J.H, S.M.J, D.J.C. B.J.M, S.F.J and G.B interpreted and analyzed the data. E.J.H and G.B performed the statistical analysis. E.J.H and G.B wrote the manuscript. All authors reviewed the manuscript.

Competing Financial Interests:

The authors declare no competing financial interests.

447 **Figure 1: The *Kcc1*^{M935K} mutation causes resistance to *P. berghei*. (A)** and **(B)** Cumulative
448 survival and average ± SEM parasitemia for WT and *Kcc1*$^{M935K/M935K}$ in male and female mice
449 respectively. WT male n=28, WT female n=8, *Kcc1*$^{M935K/M935K}$ male n=14, *Kcc1*$^{M935K/M935K}$ female
450 n=10. Combined results of two independent experiments. *P<0.05, **P<0.01, ***P<0.001.
451 P values calculated using Log rank test or the student's T-test. **(C)** average ± SEM percentage of
452 parasites which are TUNEL positive. **(D)** Average ± SEM fold change in parasitemia of
453 *Kcc1*$^{M935K/M935K}$ labelled cells compared to WT labelled cells injected into the same *P. berghei*
454 infected host (n=8)

455

456 **Figure 2: The *Kcc1*^{M935K} mutation causes resistance to cerebral malari**a. **(A)** Clinical score
457 for WT (n=37) and *Kcc1*$^{M935K/M935K}$ (n=24) mice infected with *P. berghei* 0= no symptoms, 1=
458 reduced movement, 2= rapid breathing/hunched posture, 3= ruffled fur/external bleeding, 4=
459 fitting/coma, 5= death. **(B)** Amount of Evan's Blue dye extracted from *P. berghei* infected
460 *Kcc1*$^{M935K/M935K}$ (n=8), WT (n=7) and uninfected (n=4) brains. **(C)** Representative brains dissected
461 from mice injected with Evan's Blue dye. Values are average ± SEM. *P<0.05, **P<0.01,
462 ***P<0.001. P values calculated using the Student's T-test.
463

464 **Figure 3: The *Kcc1*^{M935K} mutation does not alter immune cell populations (A)** Average ±
465 SEM proportion of lymphocytes that are CD3+, CD11c+, and CD19+ in the blood spleen and
466 thymus. **(B)** Average ± SEM proportion of CD3+ cells that are CD4+, CD8+, and CD4+CD8+ in the
467 blood spleen and thymus. Uninfected WT (n=5), uninfected *Kcc1*$^{M935K/M935K}$ (n=5), infected WT
468 (n=3), infected *Kcc1*$^{M935K/M935K}$ (n=3).
469

470 **Figure 4: The *Kcc1*^{M935K} mutation alters the inflammatory response to *P. berghei* infection**.
471 Average ± SEM concentration of cytokines in the plasma of WT (n=3-5)and *Kcc1*$^{M935K/M935K}$
472 (n=3-5) mice during infection with *P. berghei*. **P<0.01, Significance calculated using the
473 Student's T test.
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483 **Figure 1**

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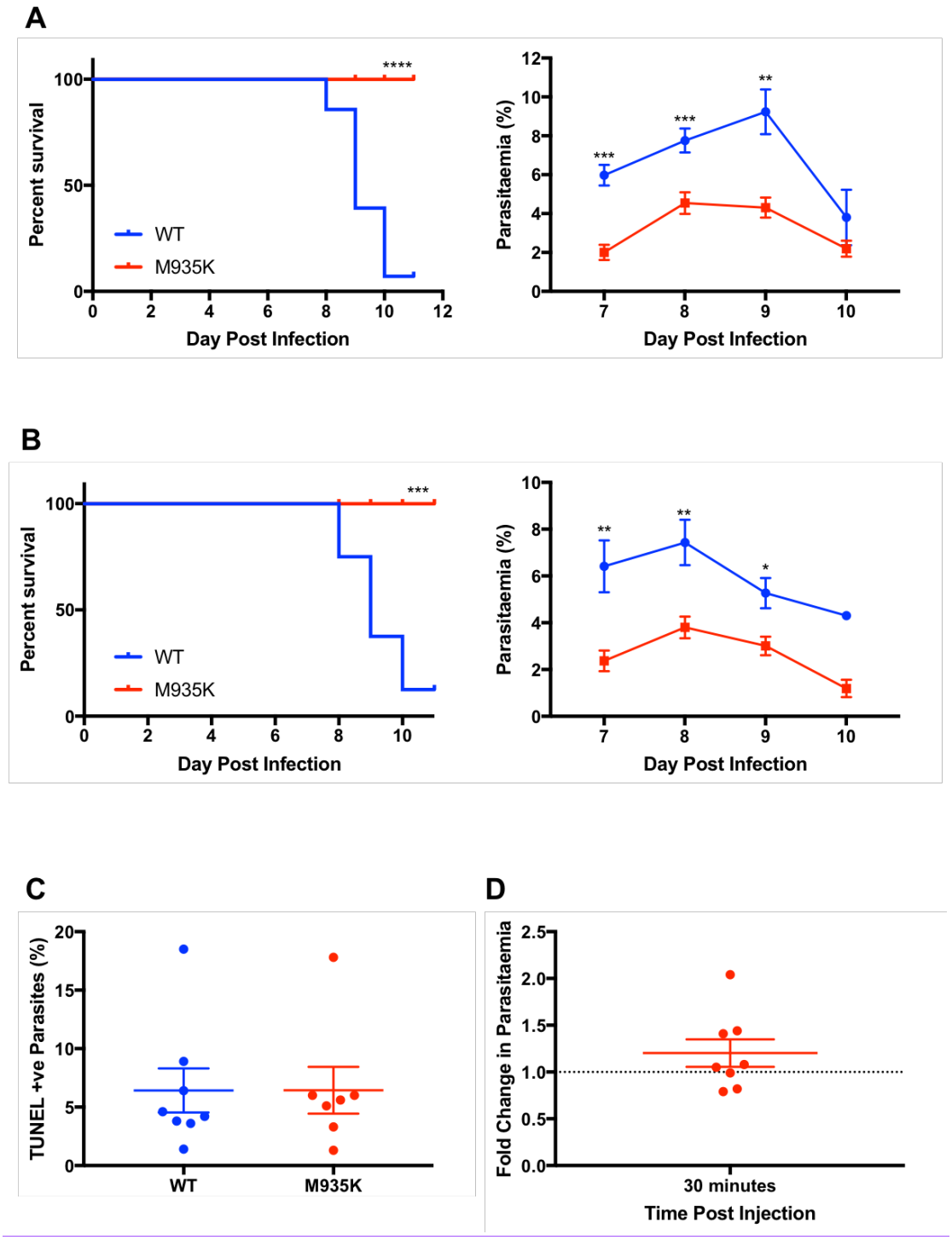

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487 **Figure 2**

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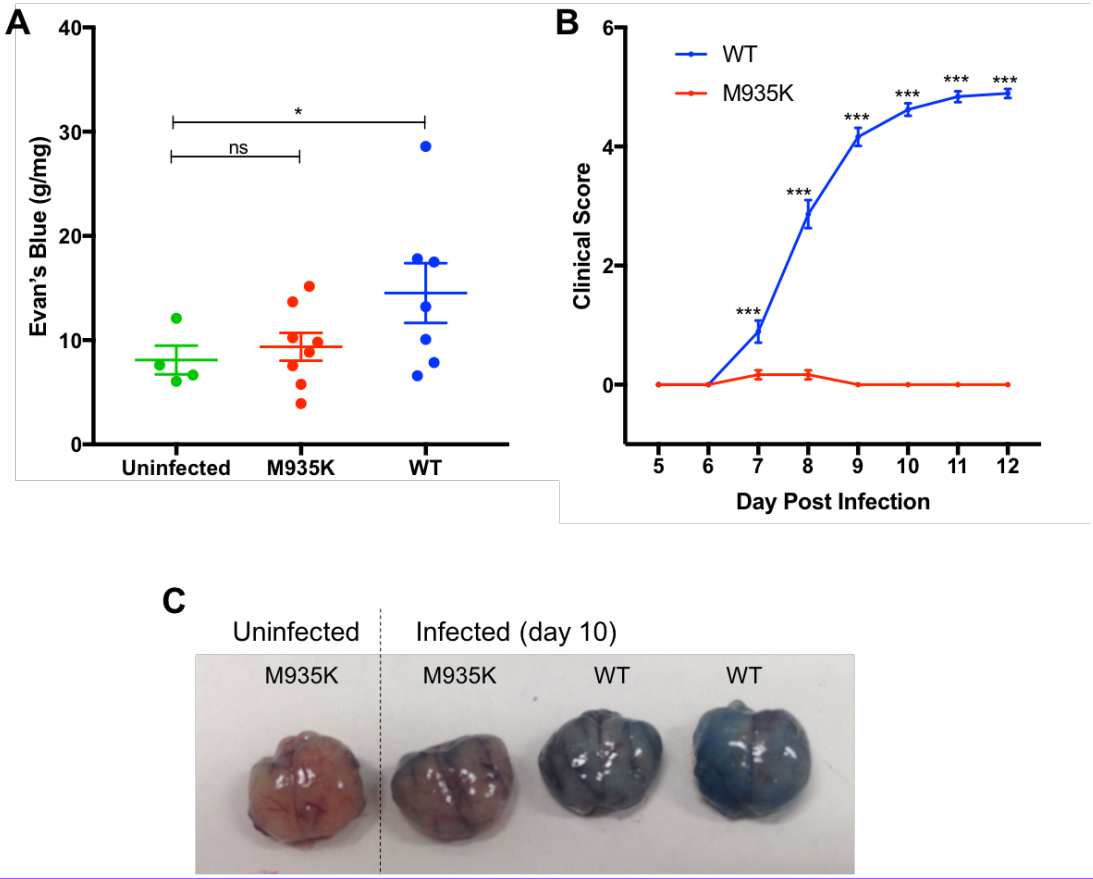

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493 **Figure 3**

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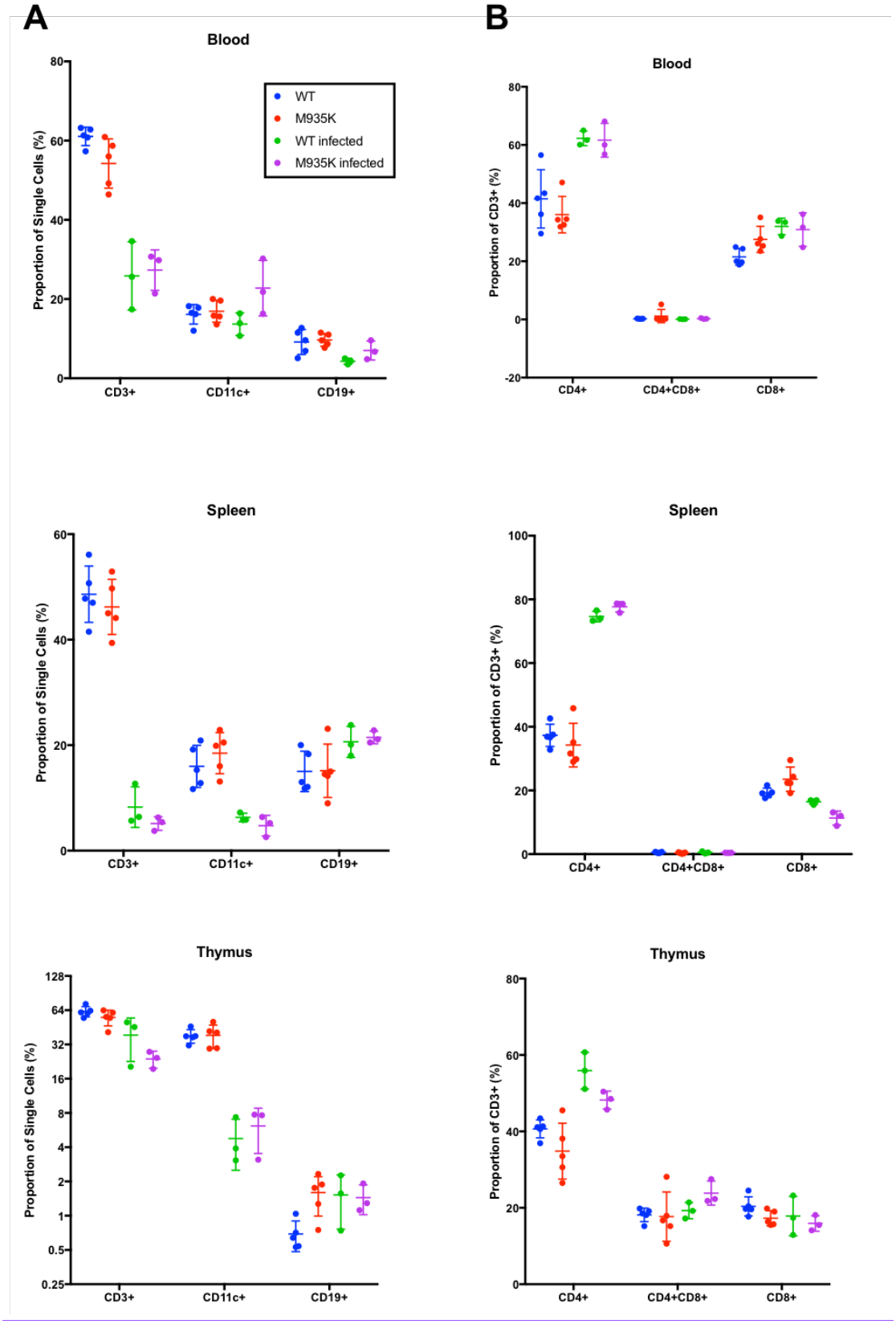

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498 **Figure 4**

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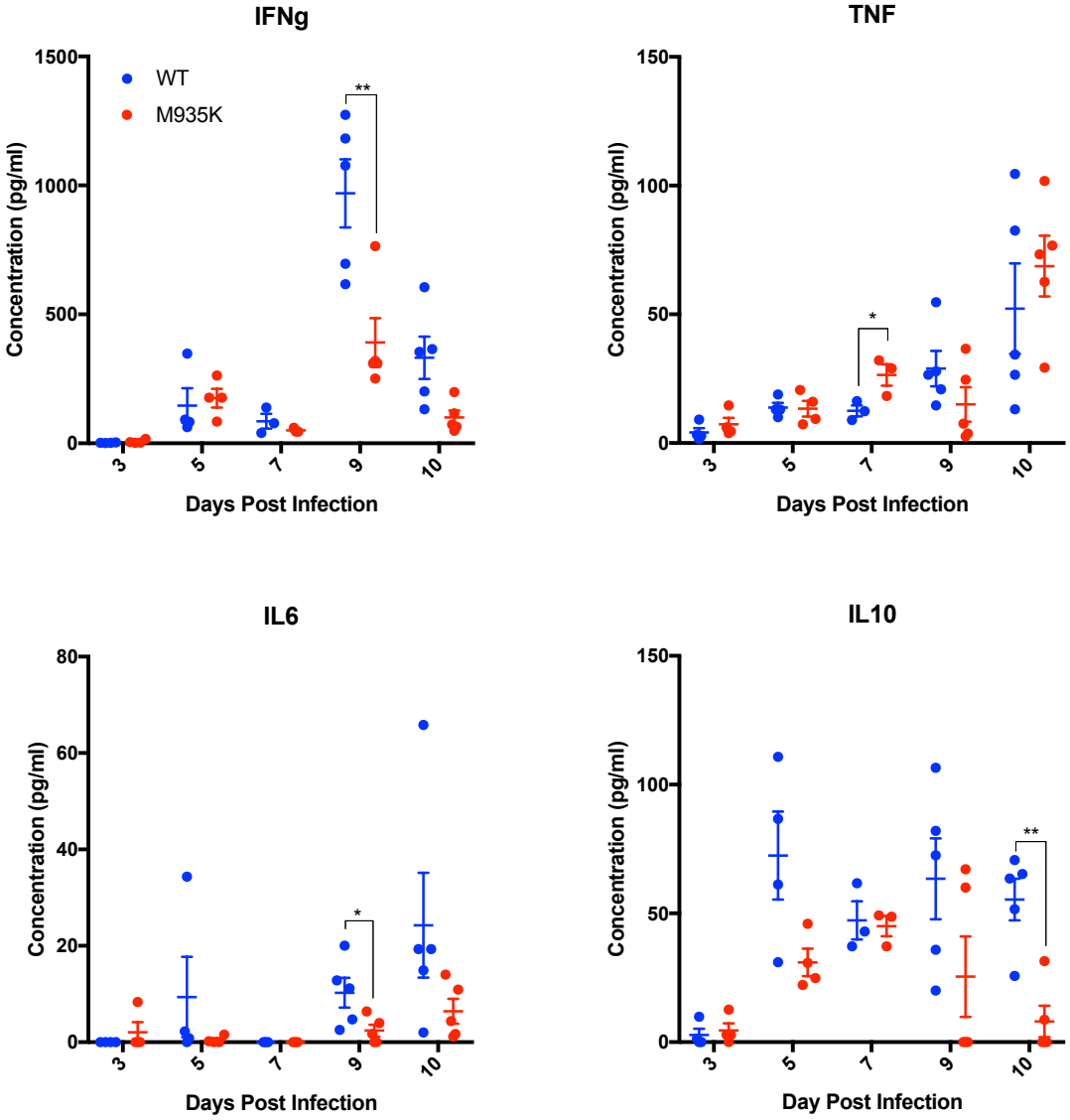

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