

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

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24

## 25 **Abstract**

26

27 *Plasmodium falciparum* malaria causes half a million deaths per year, with up to  
28 9% of this mortality caused by cerebral malaria (CM). One of the major processes  
29 contributing to the development of CM is an excess of host inflammatory  
30 cytokines. Recently K<sup>+</sup> signaling has emerged as an important mediator of the  
31 inflammatory response to infection; we therefore investigated whether mice  
32 carrying an ENU-induced activation of the electroneutral K<sup>+</sup> channel KCC1 had  
33 an altered response to *Plasmodium berghei*. Here we show that *Kcc1*<sup>M935K/M935K</sup>  
34 mice are protected from the development of experimental cerebral malaria, and  
35 that this protection is associated with an attenuated INF- $\gamma$  response. This is the  
36 first description of a K<sup>+</sup> channel affecting the development of cerebral malaria.

37

## 38 **Introduction**

39

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

48

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

58

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

69

70 Here we use the *Kcc1*<sup>M935K</sup> mice line to investigate the effect of increased host K<sup>+</sup>  
71 efflux on susceptibility to malaria infection. When *Kcc1*<sup>M935K</sup> mice were infected  
72 with *Plasmodium berghei*, they showed a marked protection from the  
73 development of experimental cerebral malaria (ECM), associated with a  
74 significant decrease in IFN $\gamma$  and IL-10 during infection, suggesting K<sup>+</sup> efflux  
75 through KCC1 attenuates the inflammatory response to infection. This is the first  
76 description of a cation transporter affecting the development of ECM.

77

78

79

## 80 **Results**

81

82 *Kcc1*<sup>M935K</sup> has increased survival to *P. berghei* challenge

83 *Kcc1*<sup>M935K/M935K</sup> mice were inoculated with *P. berghei* to determine their  
84 resistance to infection. Cumulative survival and peripheral parasitemia were  
85 monitored daily over the course of infection. When mice were infected with  
86  $1 \times 10^4$  *P. berghei* parasitized red cells, survival was significantly increased in the  
87 mutants, with 100% of homozygotes surviving past day 10 of infection,  
88 compared to 7% of WT females (P=0.0004; Figure 1A), and 11% of WT males  
89 (P=2.66x10<sup>-7</sup>; Figure1B). Significantly lower parasitemia was observed in both  
90 *Kcc1*<sup>M935K</sup> 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*<sup>M935K/M935K</sup> 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<sup>15</sup>. No  
97 significant differences were observed in either assay (Figure 1C and D),  
98 suggesting the *Kcc1*<sup>M935K</sup> mutation does not affect parasite invasion or growth.

99

#### 100 *Kcc1*<sup>M935K</sup> 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*<sup>M935K/M935K</sup>  
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*<sup>M935K/M935K</sup> 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*<sup>M935K/M935K</sup> mice are resistant to the development of ECM.

117

#### 118 *Kcc1*<sup>M935K</sup> has an abnormal immune response to infection

119

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

123 KCC1 is expressed ubiquitously<sup>20</sup>, it was hypothesized that the *Kcc1*<sup>M935K</sup>  
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<sup>21,22</sup>. 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*<sup>M935K/M935K</sup> 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*<sup>M935K/M935K</sup>. 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- $\gamma$ <sup>23,24</sup> and TNF $\alpha$ <sup>25</sup>, and can be  
147 induced by depletion of the anti-inflammatory cytokine IL-10<sup>26</sup>. CM resistance is  
148 also observed in mice with defective T cell dependent IFN- $\gamma$  production<sup>19</sup>. 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- $\gamma$  and TNF $\alpha$  over the  
153 course of infection, with IFN- $\gamma$  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*<sup>M935K</sup> mice displayed similar trends, but differed significantly

156 from WT in several key points. On day 7 post infection, *Kcc1*<sup>M935K</sup> showed a 1.2-  
157 fold increase in TNF $\alpha$ , which was followed on day 9 by a 0.6-fold reduction in the  
158 amount of IFN- $\gamma$ , and a 0.8-fold reduction in the amount of IL-6 compared to WT.  
159 On day 10 of infection *Kcc1*<sup>M935K</sup> 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<sup>27,28</sup>, or erythrocyte membrane proteins<sup>29-31</sup>.

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*<sup>M935K/M935K</sup> 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<sup>32</sup>. 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<sup>23-25</sup> were shown to be attenuated

188 in the mutant animals, particularly IFN- $\gamma$  and IL10. It is not clear from this study  
189 exactly what is causing decreased INF- $\gamma$  and IL-10 in *Kcc1*<sup>M935K</sup>. 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<sup>+</sup> efflux can alter  
193 cellular cytokine production<sup>5-7</sup>; can increase assembly of the NALP  
194 inflammasome in response to pathogen associated proteins<sup>7</sup>; and is essential  
195 macrophage migration<sup>33</sup>. All of these may help contribute to both the attenuated  
196 inflammatory response, and the lower parasitemia observed in *Kcc1*<sup>M935K</sup> 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 achievable<sup>34,35</sup>, 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*<sup>M935K</sup> mutation is carried on a mixed BALB/c and C57BL/6 background<sup>14</sup>.  
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*<sup>M935K/M935K</sup> x *Kcc1*<sup>M935K/M935K</sup> 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*<sup>M935K</sup> was periodically crossed back to WT, and new WT x WT and  
218 *Kcc1*<sup>M935K/M935K</sup> x *Kcc1*<sup>M935K/M935K</sup> 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<sup>36</sup>. Experimental mice were infected intraperitoneally at a dose of  
224  $1 \times 10^4$  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 200 $\mu$ l 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. 200 $\mu$ l 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 40 $\mu$ g/ml to 0 $\mu$ g/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 schizogony, 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<sup>6</sup>  
316 cells were collected for each sample, and data was analysed using FlowJo (FlowJo,  
317 LLC, Oregon, USA).

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## 322 References

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- 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-mediated 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).

- 418 34 Delpire, E. & Kahle, K. T. The KCC3 cotransporter as a therapeutic target  
419 for peripheral neuropathy. *Expert Opin Ther Targets* **21**, 113-116, (2017).  
420 35 Yamada, K. *et al.* Small-molecule WNK inhibition regulates cardiovascular  
421 and renal function. *Nat Chem Biol* **12**, 896-898, (2016).  
422 36 Lelliott, P. M., Lampkin, S., McMorran, B. J., Foote, S. J. & Burgio, G. A flow  
423 cytometric assay to quantify invasion of red blood cells by rodent  
424 *Plasmodium* parasites in vivo. *Malar J* **13**, 100, (2014).  
425

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436

#### 437 **Author Contribution Statement:**

438

439 E.J.H, B.J.M, S.F.J and G.B designed and planed the experimental work. E.J.H, and  
440 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  
441 and analyzed the data. E.J.H and G.B performed the statistical analysis. E.J.H and  
442 G.B wrote the manuscript. All authors reviewed the manuscript.

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#### 444 **Competing Financial Interests:**

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446 The authors declare no competing financial interests.

447 **Figure 1: The *Kcc1*<sup>M935K</sup> mutation causes resistance to *P. berghei*.** (A) and (B) Cumulative  
448 survival and average  $\pm$  SEM parasitemia for WT and *Kcc1*<sup>M935K/M935K</sup> in male and female mice  
449 respectively. WT male n=28, WT female n=8, *Kcc1*<sup>M935K/M935K</sup> male n=14, *Kcc1*<sup>M935K/M935K</sup> 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  $\pm$  SEM percentage of  
452 parasites which are TUNEL positive. (D) Average  $\pm$  SEM fold change in parasitemia of  
453 *Kcc1*<sup>M935K/M935K</sup> labelled cells compared to WT labelled cells injected into the same *P. berghei*  
454 infected host (n=8)

455

456 **Figure 2: The *Kcc1*<sup>M935K</sup> mutation causes resistance to cerebral malaria.** (A) Clinical score  
457 for WT (n=37) and *Kcc1*<sup>M935K/M935K</sup> (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*<sup>M935K/M935K</sup> (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  $\pm$  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*<sup>M935K</sup> mutation does not alter immune cell populations** (A) Average  $\pm$   
465 SEM proportion of lymphocytes that are CD3+, CD11c+, and CD19+ in the blood spleen and  
466 thymus. (B) Average  $\pm$  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*<sup>M935K/M935K</sup> (n=5), infected WT  
468 (n=3), infected *Kcc1*<sup>M935K/M935K</sup> (n=3).

469

470 **Figure 4: The *Kcc1*<sup>M935K</sup> mutation alters the inflammatory response to *P. berghei* infection.**  
471 Average  $\pm$  SEM concentration of cytokines in the plasma of WT (n=3-5) and *Kcc1*<sup>M935K/M935K</sup>  
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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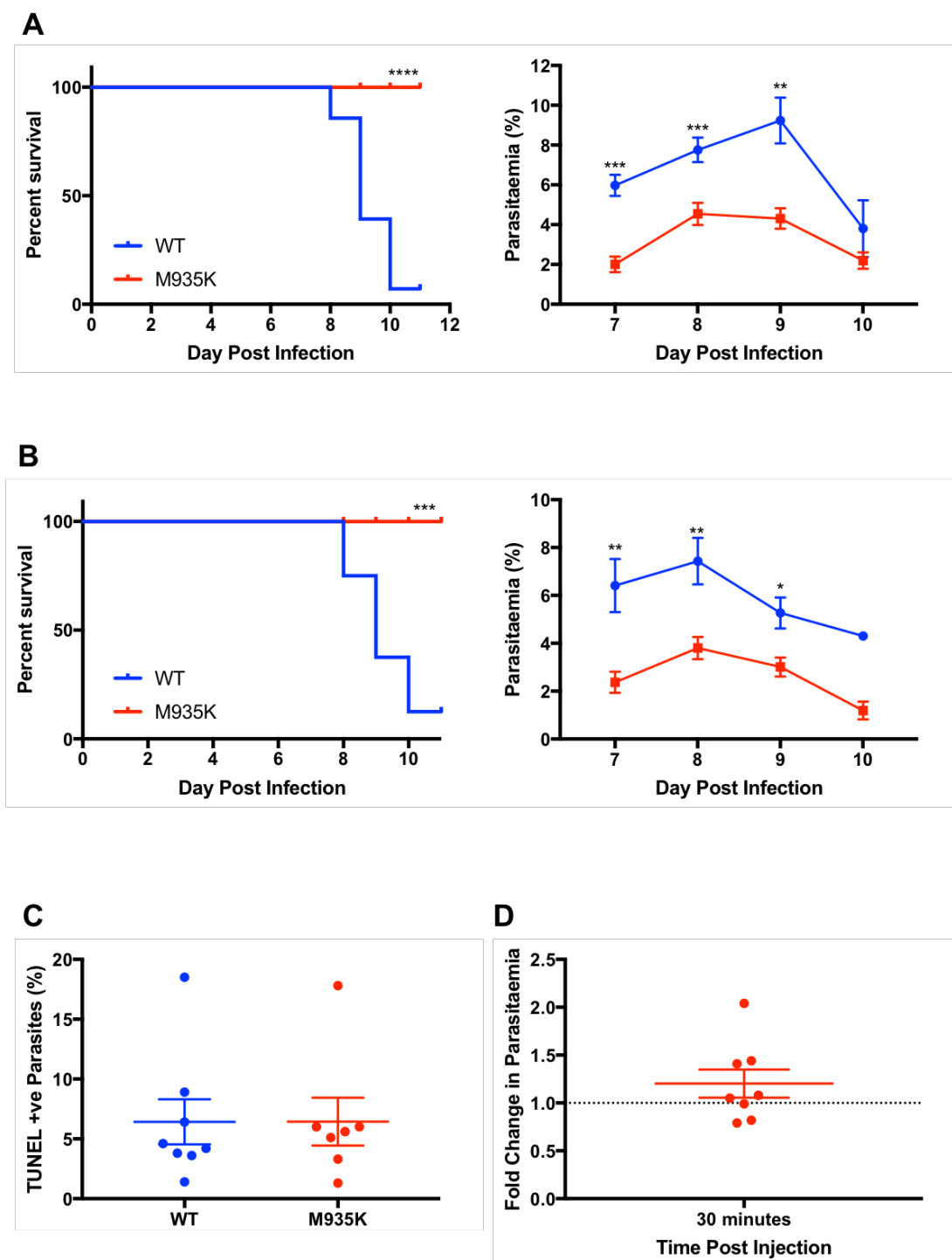
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483 **Figure 1**

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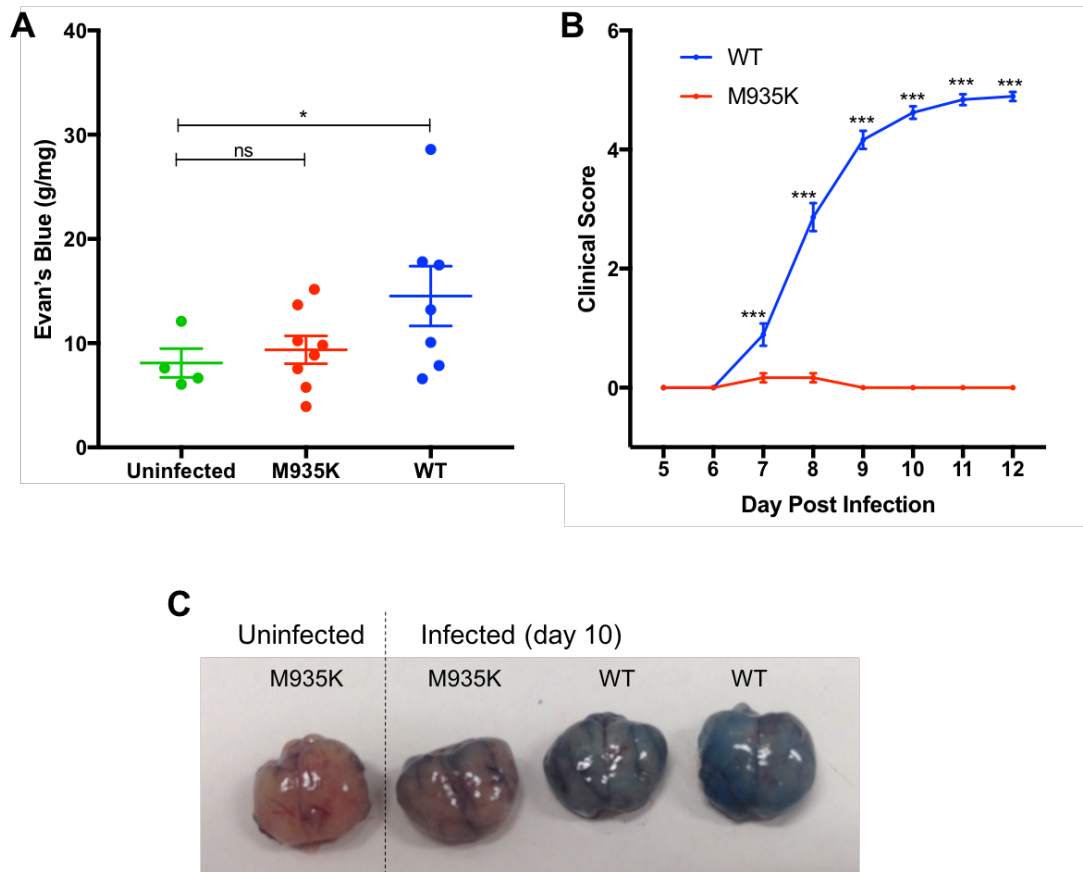


487 **Figure 2**

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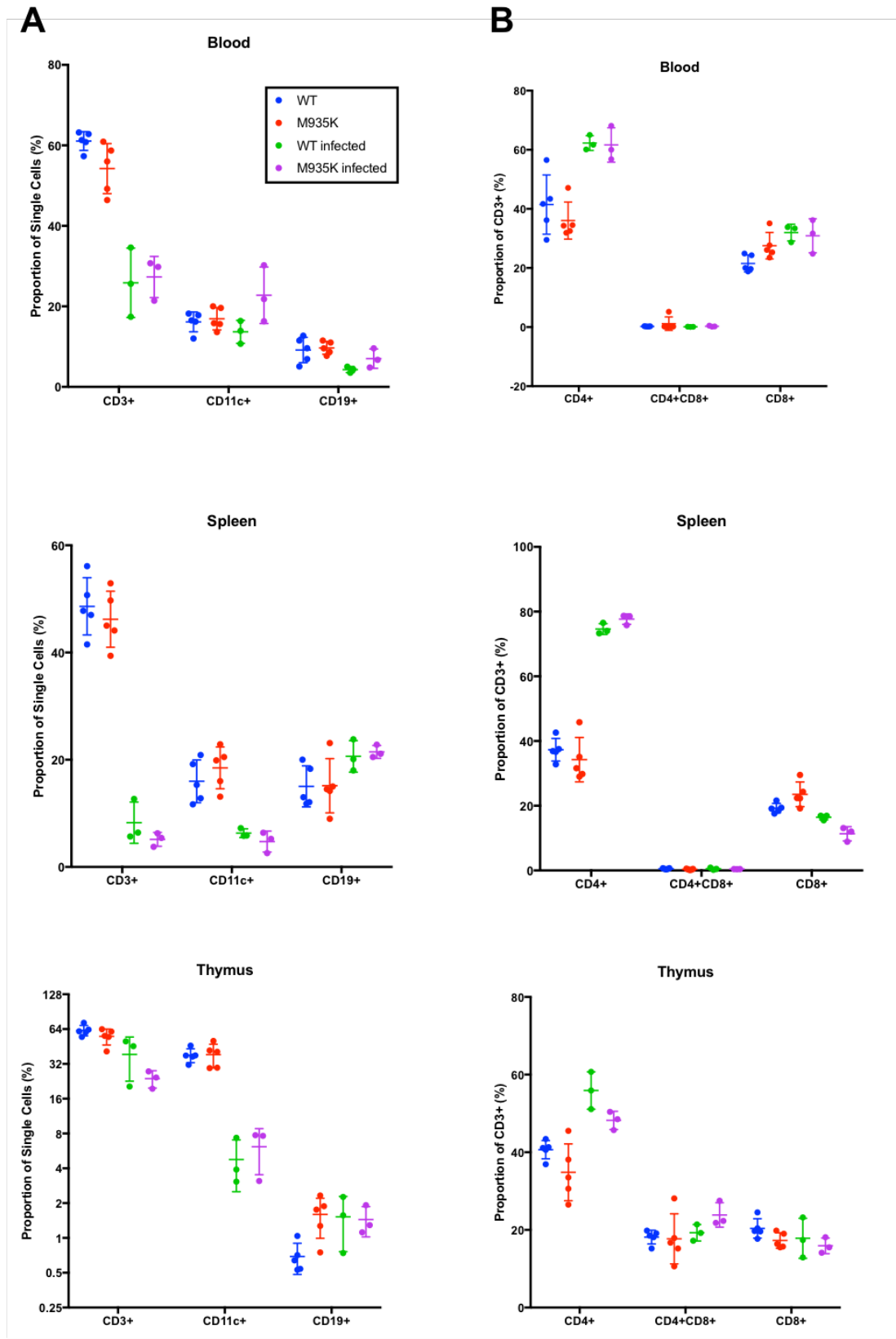


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493 **Figure 3**

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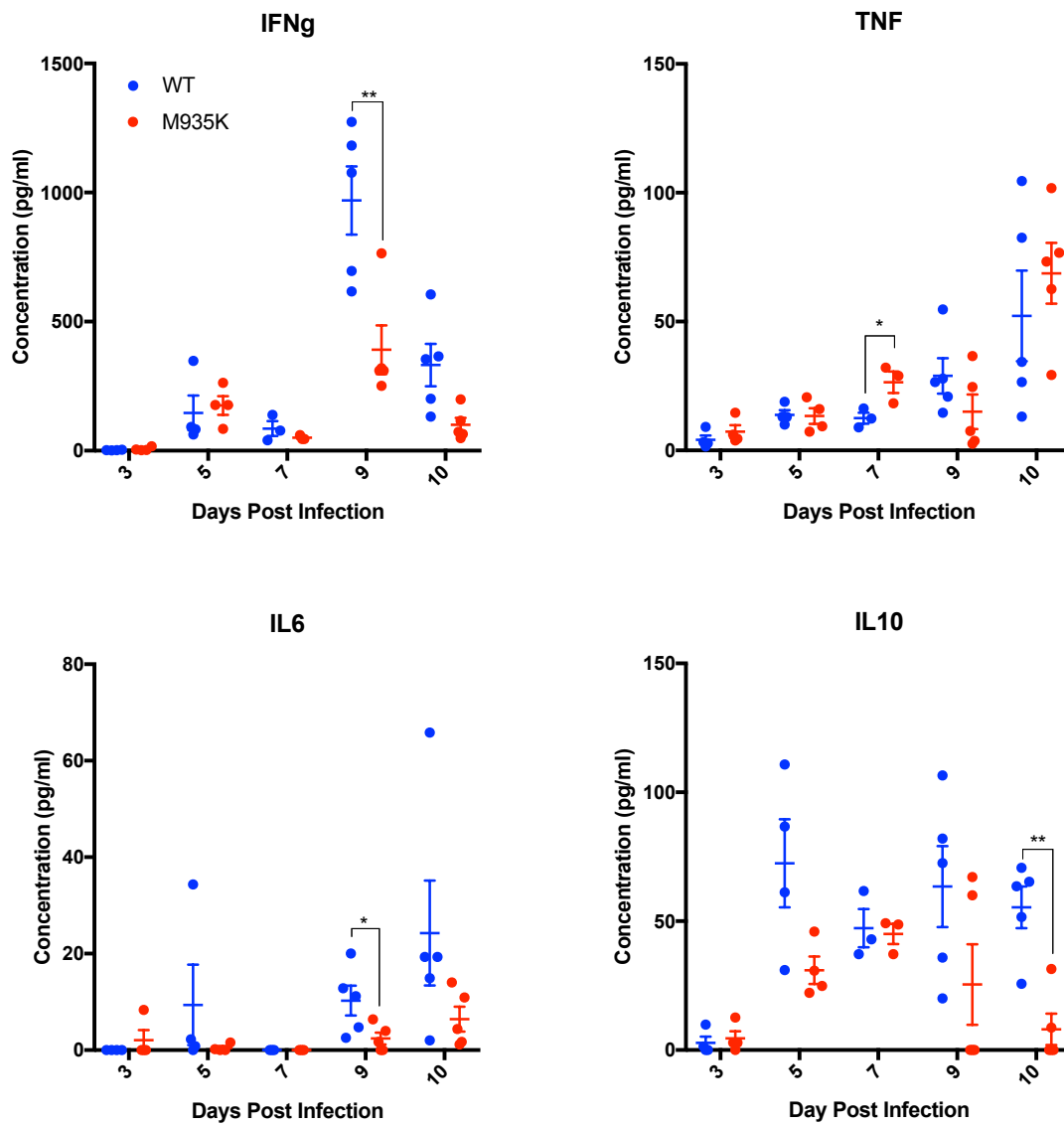
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498 **Figure 4**

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