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

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

Kcc1^{M935K} *is resistant to ECM*

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

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

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

KCC1 is expressed ubiquitously[20], it was hypothesized that the *Kcc1*^{M935K} mutation might cause alterations to some of these immune cell populations. Therefore, the relative proportion of CD3+, CD11c+, and CD19+ cells were measured by flow cytometry in the blood, spleen and thymus, both in non-infected mice, and day 8 of *P. berghei* infection where the inflammatory response is expected to be highest[21,22]. No significant differences in these cell populations were observed in any of these tissues, before or during infection (Figure 3). CD3+ cells were further analyzed to determine the relative proportion of CD4+ and CD8+ subsets. Again, no significant differences were observed. In WT mice the average proportion of CD4+ cells rose with infection in the blood, spleen, and thymus, from 41 to 62%, 37 to 75%, and 40 to 56% respectively. This trend was also observed in *KCC1*$^{M935K/M935K}$ where CD4+ cells rose from an average proportion of 36 to 62%, 34 to 78%, and 35 to 48% respectively (Figure 3). The average proportion of CD8+ also increased in the blood with infection, from 22 to 32% in WT mice, and from 28 to 31% in *KCC1*$^{M935K/M935K}$. Conversely, in both the spleen and thymus the average proportion of CD8+ cells was similar between uninfected and infected animals. Together we noted no difference in T-cell response.

One of the major host processes known to contribute to the development of cerebral malaria in *P. berghei* infection is an over-active cytokine-driven inflammatory response. Both *in vivo* neutralisation of host molecules, and studies with knock-out mice have shown that cerebral malaria can be prevented by depletion of the pro-inflammatory cytokines IFN-γ [23,24] and TNFα [25], and can be induced by depletion of the anti-inflammatory cytokine IL-10 [26]. CM resistance is also observed in mice with defective T cell dependent IFN-γ production [19]. We therefore measured plasma cytokine levels in infected mice over the course of *P. berghei* infection.

As expected, WT mice showed strong increases in both IFN-γ and TNFα over the course of infection, with IFN-γ peaking around the onset of symptoms at day 9. IL-10 was variable over the course of infection, and IL-6 showed a smaller, late 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

in the mutant animals, particularly IFN-γ and IL10. It is not clear from this study exactly what is causing decreased INF-γ and IL-10 in *Kcc1*^{M935K}. This reduced inflammation may simply be the result of the lower parasitemia observed in mutants. However, KCC1 is expressed on a wide range of immune cells, and this may affect their function. Previous studies have shown that K+ efflux can alter cellular cytokine production[5-7]; can increase assembly of the NALP inflammasome in response to pathogen associated proteins[7]; and is essential macrophage migration[33]. All of these may help contribute to both the attenuated inflammatory response, and the lower parasitemia observed in *Kcc1*^{M935K} mice.

Here we have shown that activation of KCC1 causes a dramatic resistance to *P. berghei* by completely preventing the development of experimental cerebral malaria (ECM). This is the first description of a mutation in a transporter that has an effect on ECM. Previous studies have shown that pharmacological activation of KCC channels is achiavable[34,35], therefore future research into KCC1 activation may provide novel treatments for cerebral malaria.

Methods

Animals

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

Infections

Experiments used either the rodent parasite *P. berghei ANKA*. Parasite stocks were prepared from passage through resistant SJL/J mice, as described previously[36]. Experimental mice were infected intraperitoneally at a dose of $1x10^4$ parasitised RBC. Blood stage parasitemia was determined by counting thin smears from tail blood stained in 10% Giemsa solution. A least 300 cells were counted per slide.

Histology

Thin tail smears from *P. berghei* infected mice were fixed in 100% MeOH, and stained with an APO-BrdU TUNEL assay kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Slides were examined on an upright epifluorescence microscope (ZIESS) 600x magnification. 10 fields of view were counted for each slide.

Evans Blue

P. berghei infected mice, and uninfected controls, were injected IV with 200µl 1% Evans Blue/PBS solution. 1hr post injection, mice were sacrificed and their brains collected and weighed. Brains were placed in 2ml 10% neutral buffered formalin at room temperature for 48hrs to extract dye. 200µl of formalin from each brain was then collected and absorbance measured at 620nm. Amount of Evans blue extracted per gram brain tissue was calculated using a standard curve ranging from 40µg/ml to 0µg/ml. Injections were carried out on the day of infection that the first mouse died.

Clinical Score

Mice were monitored three times daily, and given a score from 0 to 5 based on the type and severity of their symptoms. '0' indicated no symptoms; '1' reduced or languid movement; '2' rapid breathing and/or hunched posture; '3' ruffled fur, dehydration and/or external bleeding; '4' fitting and/or coma; '5' death. Mice were considered comatose if they were unable to right themselves after being placed on their side. The highest score recorded for each mouse on each day was used to generate daily averages.

Cytokines

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

Lymphocyte Analysis

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

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

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

TUNEL staining

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

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

In-vivo Invasion Assay

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

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

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

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

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Acknowledgement

We would like to acknowledge Shelley Lampkin and Australian Phenomics Facility (APF) for the maintenance of the mouse colonies. This study was funded by the National Health and Medical Research Council of Australia (Program Grant 490037, and Project Grants 605524 and APP1047090), the National Collaborative Research Infrastructure Strategy (NCRIS), the Education Investment Fund from the Department of Education and Training, the Australian Phenomics Network, Howard Hughes Medical Institute and the Bill and Melinda Gates Foundation.

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.

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

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

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

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

483 **Figure 1**

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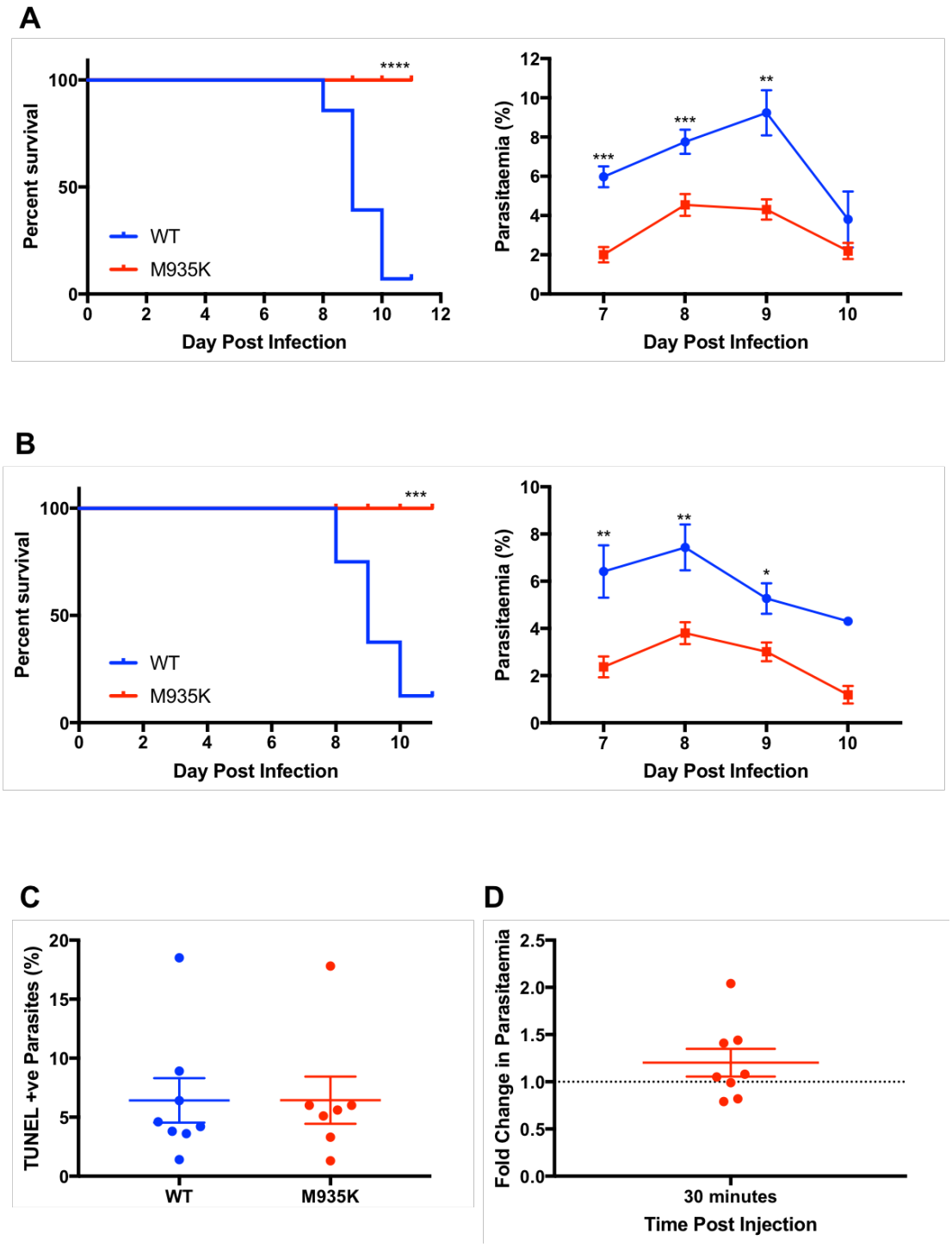

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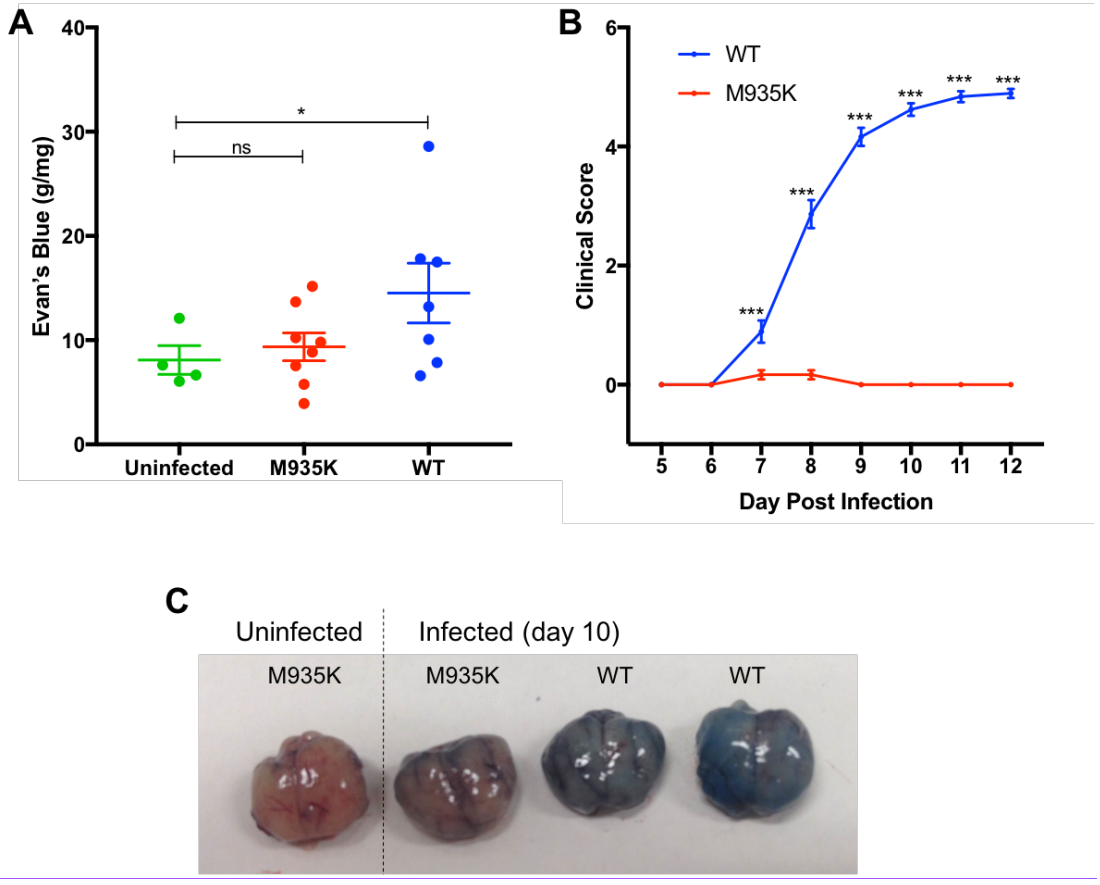
Figure 2



493 **Figure 3**

494

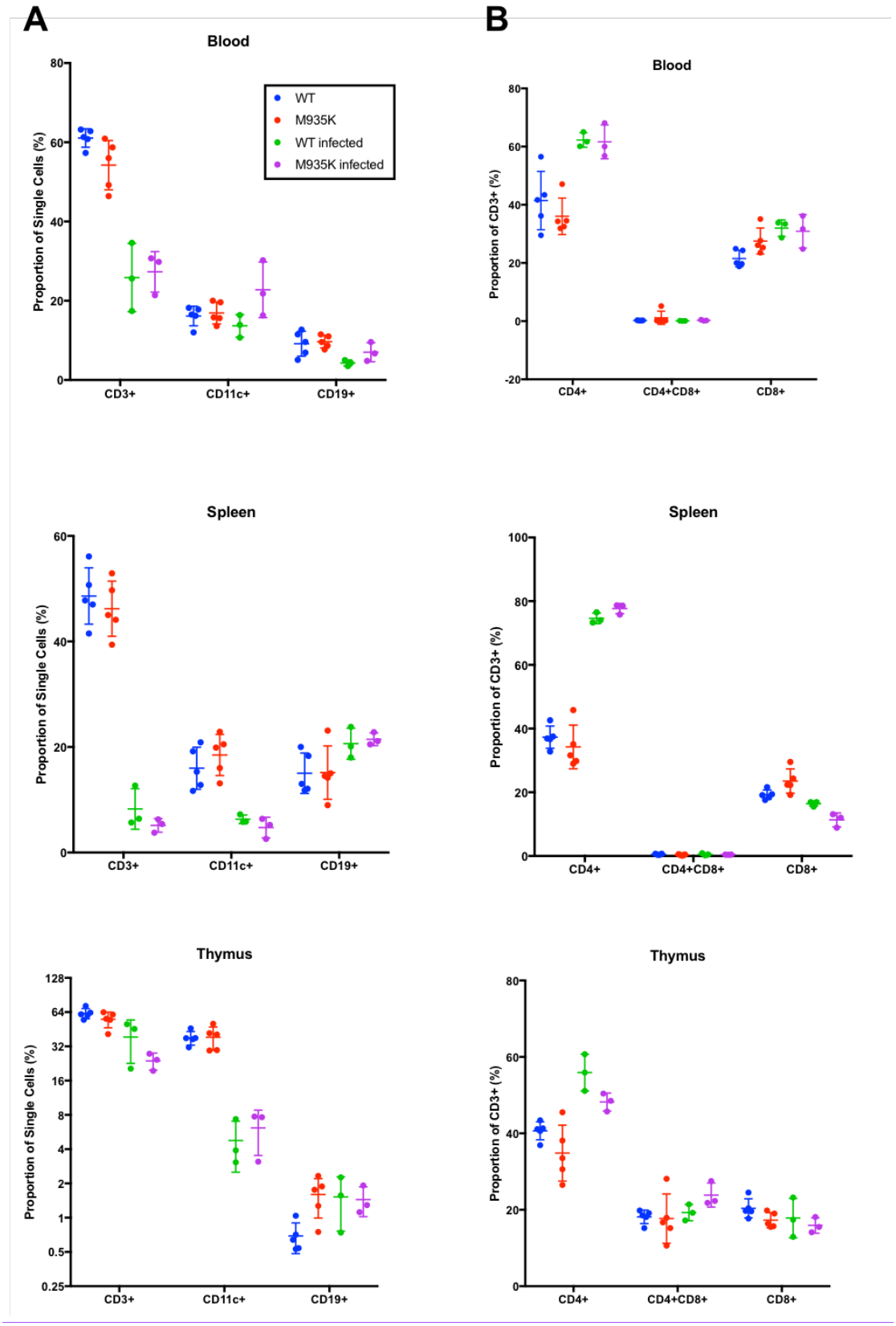


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Figure 4