Analysis of Spleen Histopathology, Splenocyte composition and Hematological Parameters in Mice Infected with *Plasmodium berghei* K173

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ABSTRACT Malaria is a fatal disease that presents clinically as a continuum of symptoms and severity, which are determined by complex host-parasite interactions. Clearance of infection is believed to be accomplished by the spleen and mononuclear phagocytic system (MPS), both in the presence and absence of artemisinin treatment. The spleen filters infected RBCs from circulation through immune-mediated recognition of the infected RBCs followed by phagocytosis. Using different strains of mice infected with *P. berghei* K173 (PbK173), the mechanisms leading to splenomegaly, histopathology, splenocyte activation and proliferation, and their relationship to control of parasitemia and host mortality were examined. Survival time of mice infected with PbK173 varied, although the infection was uniformly lethal. Mice of the C57BL/6 strain were the most resistant, while mice of the strain ICR were the most susceptible. BALB/c and KM mice were intermediate. In the course of PbK173 infection, both strains of mice experienced significant splenomegaly.

30   Parasites were observed in the red pulp at 3 days post infection in all animals. All

31   spleens retained late trophozoite stages as well as a fraction of earlier ring-stage

32   parasites. The percentages of macrophages in infected C57BL/6 and KM mice were

33   higher than uninfected mice on 8 dpi. Spleens of infected ICR and KM mice exhibited

34   structural disorganization and remodeling. Furthermore, parasitemia was significantly

35   higher in KM versus C57BL/6 mice at 8 dpi. The percentages of macrophages in ICR

36   infected mice were lower than uninfected mice, and the parasitemia was higher than

37   other strains. The results presented here demonstrate the rate of splenic mechanical

38   filtration and the splenic macrophages likely contribute to an individual's total

39   parasite burden. This in turn can influence the pathogenesis of malaria. Finally,

40   different genetic backgrounds of mice have different splenic mechanisms for

41   controlling malaria infection.

42   **KEYWORDS** Malaria, control infection, spleen filtration, splenocyte

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45   *Plasmodium falciparum* parasitescause lethal infections worldwide, especially in

46   Africa (1). Reducing this disease burden continues to rely heavily on the availability

47   and proper use of effective antimalarial drugs. Artemisinin and its derivatives are

48   sesquiterpene lactones with potent activity against nearly all blood stages of *P.*

49   *falciparum*. There is a natural and complex variation in the pathogenesis and clinical

50   presentation of malaria, which is influenced by host age, immunity and genetic

51   background, as well as by environmental conditions and parasite genetics (2,3). Host

52   immunity and genetic factors are estimated to account for one quarter of the total

53   variability in malaria severity (4,5). Host defense mechanisms such as removal of

54   circulating parasites by the spleen and mononuclear phagocytic system (MPS) are

55   thought to play a major role in rapid control of infection (6), in the presence or

56   absence of artemisinin treatment (7).

57   The function of the spleen is to remove senescent erythrocytes (RBCs) and

58   circulating foreign material such as bacteria or cellular debris (8). The structure of the

59   spleen is complex with 2 overlapping blood circulations—a rapid flow by-pass, called

60   the fast closed circulation, which accommodates roughly 90% of the splenic blood

61   flow (100–300 mL/min in a healthy adult), and a slow open circulation in which the

62   blood is filtered through narrow inter-endothelial slits (9,10). In the slow open

63   microcirculation, RBCs navigate through the cords of the red pulp before returning to

64   the vascular beds by squeezing between endothelial cells in the sinus walls (11-13).

65   Crossing splenic inter-endothelial slits poses the greatest demand on RBC

66   deformability in the body (14) and is believed to result in the retention of less

67   malleable RBCs or in removal of intraerythrocytic bodies. In malaria, the spleen

68   filters infected RBCs from circulation by physical selection as well as

69   immune-mediated recognition and phagocytosis of infected RBCs (15). These

70   processes play a central role in the clearance of circulating malaria parasites (6). The

71   rate of splenic mechanical filtration may be one factor affecting an individual's total

72   parasite burden and the pathogenesis of malaria. Understanding the role of the spleen

73   in host defense may shed additional light on the variation in human susceptibility to

74   malaria and offer insights into possible mechanisms of malaria pathogenesis.

75   In the present study, the host defense against blood-stage malaria was examined by

76   using different strains of mice infected with P. *berghei* K173 (PbK173), a rodent-lethal

77   strain of malaria. Parasitemia and survival were measured to monitor the course of

78   infection in C57BL/6, BALB/C, ICR, and KM mice. Since C57BL/6 mice were found

79   to be more resistant to this infection, parameters indicative of a protective host

80   response to infection were also characterized in the four strains mice. These included

81   splenomegaly, histopathology, splenocyte subsets, hematological parameters.

82   **RESULTS**

83   **Progression of infection with PbK173 in mice**

84   Mice (18~22g) were purchased from Weitonglihua, and were inoculated with 10^7 P.

85   *berghei* K173 infected erythrocytes in 200 μL of sterile saline buffer i.p. Mice were

86   monitored daily for any signs of malaria, including parasitemia and behavioral

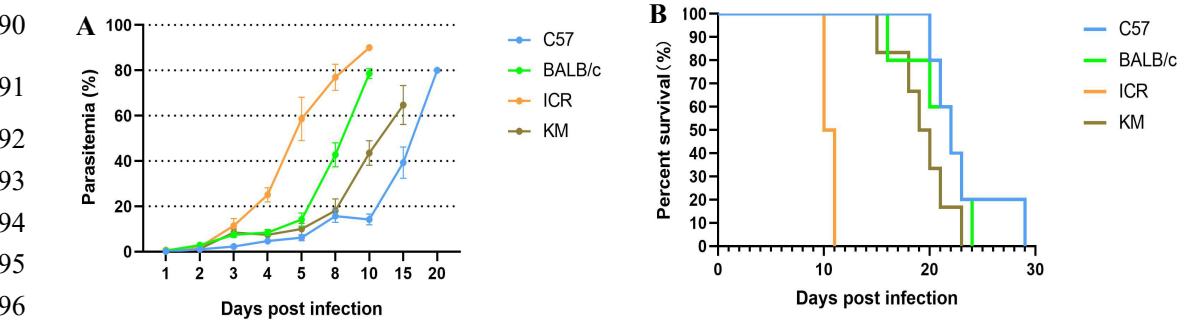87   changes, compared to uninfected controls.

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FIG 1: Parasitemia and survival curves in C57BL/6, BALB/c, ICR and KM mice following PbK173 infection. A: The percentage of parasitemia is presented as the arithmetic mean of each mouse strain $\pm$ SD. B: Survival of mice without treatment (n=6) as a function of days post infection with PbK173.

The combination of different mouse strains and parasites resulted in different disease outcomes following infection. BALB/c, ICR and KM mice developed infection with parasites observed in the blood as early as 1 dpi, whereas C57 mice converted at 2 dpi. Parasitemia was progressive in all groups (Fig. 1A). On 5 dpi, the parasitemia in ICR mice was 58.6%, 90% on the 8 dpi, and all animals died between 9-11 dpi (Fig. 1B). In BALB/c mice, parasitemia approached 50% by 8 dpi, and peaked at 80% on 10 dpi. The BALB/c mice all succumbed between 16-24 dpi. On the 15th day, the highest parasitemia of the KM mice was 65%, and the animals all died between 15-23 dpi. The parasitemia of C57 mice was close to 50% on the 17th day, and the highest parasitemia reached 80% on the 20th day. All C57 mice died from malaria by 29 dpi.

It was observed that C57 mice were more resistant to infection than the other strains examined, as evidenced by the latest peak parasitemia and prolonged survival. The highest parasitemia of KM mice was the lowest observed, but its survival was shorter than that of C57BL/6 mice. The highest parasitemia of ICR mice was higher than other strains, although these mice succumbed the fastest.

Hematological Parameters

In this study, RBC counts (mean $\pm$ SD, 10^{12}/L) for uninfected control mice were as follows: C57BL/6 (10.01±0.25), BALB/c (7.93±1.01), ICR (8.16±0.16), KM (8.64±0.29). These values are within normal ranges, as reported previously. By 5 dpi,,

123 all strains of mice presented with anaemia, thrombocytopaenia, and leukocytosis

124 (Table 1).

125   Malaria infections induce lymphocytopenia and an increase in neutrophils, which is

126 indicative of systemic inflammation (16). In all 4 strains of mice, the percentages of

127 lymphocytes decreased compared to the baseline values starting from 1 dpi. In ICR

128 mice, the decrease progressed significantly over the first 3 days. The

129 lymphocytopenia progressed slowly in BALB/c and KM mice on the 2nd-3rd day of

130 infection (Fig. 2A). In all mice, the percentages of neutrophils increased relative to

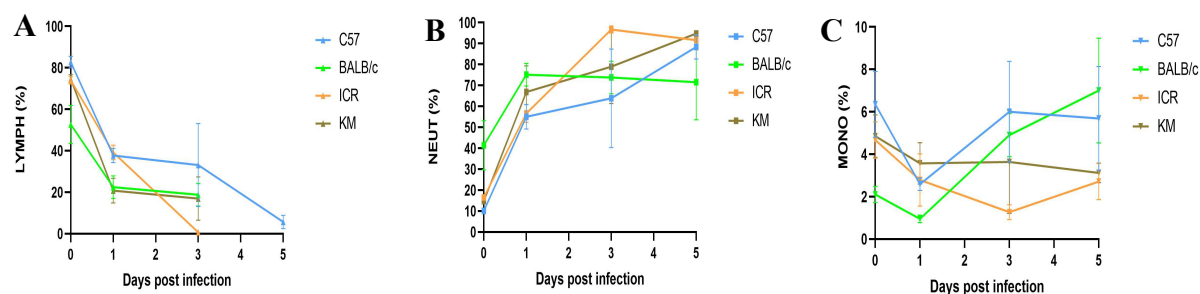131 uninfected control mice starting from 1 dpi (Fig. 2B).

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140 FIG 2: Effects of PbK173 infection on hematological parameters in C57BL/6,
141 BALB/c, ICR and KM mice. A: Lymphocytes B: Neutrophils C: Monocytes.

142

143   The percentages of monocytes decreased compared with baseline values on at 1 dpi

144 for all infected groups. But on 5 dpi, the percentages of monocytes in BALB/c

145 (7.00±2.46, $p < 0.05$) and C57BL/6 (5.68±2.45, $p > 0.05$) mice were increased.

146   In contrast, monocyte counts decreased in ICR (2.71±0.85, $p < 0.05$) and KM

147 (3.11±0.46, $p < 0.05$) mice (Fig. 2C).

Table 1: Effects of PbK173 infection on some hematological parameters in infected mice on 5 dpi.

Hematological parameters	C57BL/6		BALB/c		ICR		KM	
	Uninfected	Infected	uninfected	infected	uninfected	infected	uninfected	infected
PLT (10^9/L)	1278.83±89.60	285.00±34.88↓***	1310.50±57.76	312.33±19.59↓***	1635.67±68.14	296.00±33.80↓***	1476.17±99.99	341.33±31.87↓***
RBC (10^{12}/L)	10.01±0.25	7.75±0.62↓***	7.93±1.01	6.62±0.37↓*	8.16±0.16	3.39±0.35↓***	8.64±0.29	4.44±0.34↓***
HGB (g/L)	149.67±2.66	113.83±8.45↓***	132.33±12.07	101.50±4.42↓*	137.17±4.26	60.00±6.96↓***	129.67±5.98	68.83±4.92↓***
HCT (%)	47.23±0.96	37.78±3.03↓***	40.40±3.28	32.58±1.33↓*	42.48±1.10	21.27±2.19↓***	42.67±2.02	24.77±1.86↓***
MCV (fL)	46.52±0.43	48.40±0.71↑***	45.02±0.67	49.22±1.48↑*	51.77±1.33	62.72±3.15↑***	48.75±0.83	56.26±1.12↑***
MCH (pg)	14.71±0.07	14.56±0.24	14.73±0.15	15.46±0.63↑*	16.86±0.35	17.55±0.76	15.18±0.35	15.75±0.12↑*
MCHC (g/L)	316.83±2.04	301.17±2.14↓***	327.17±4.35	310.50±5.17↓***	321.67±3.26	283.83±5.49↓***	310.67±3.38	279.00±5.66↓***
RDW-CV (%)	17.35±.26	16.20±0.83↓*	19.03±0.99	16.72±0.92↓*	15.85±0.32	19.97±1.21↓*	16.75±0.57	15.83±1.64
RDW-SD (fL)	22.80±0.59	24.50±1.16↑*	25.82±2.22	26.87±1.49	26.70±0.84	41.11±4.13↑***	27.27±0.74	31.98±1.85↑***
WBC (10^9/L)	2.74±0.39	5.84±2.16↑*	4.37±0.93	8.64±1.95↑*	2.52±0.35	5.63±1.64↑*	1.65±0.30	3.45±0.43↑***
NEUT (%)	10.13±0.4	88.33±5.83↑***	32.68±9.50	76.53±10.66↑***	16.50±1.07	91.65±2.59↑***	14.78±1.31	94.77±1.04↑***
LYMPH (%)	82.80±2.78	5.71±3.13↓***	52.68±9.16	---↓***	73.98±1.77	---↓***	74.57±2.09	---↓***
MONO (%)	6.35±1.55	5.68±2.45	2.10±0.38	7.00±2.46↑*	4.66±0.85	2.71±0.85↓*	4.85±1.01	3.11±0.46↓*
EO (%)	1.47±0.67	0.47±0.38↓*	2.13±0.59	1.33±0.68	4.06±0.53	3.98±2.73	4.60±1.66	0.71±0.24↓*
BASO (%)	0.32±0.23	0.63±0.23	0.16±0.05	0.38±0.04↑*	0.25±0.20	0.45±0.08	0.51±0.17	0.58±0.20

Values are given as mean ± SD, n=6. * and *** indicate $p < 0.05$ and $p < 0.001$ compared with the uninfected group, respectively

Gross and histopathologic analysis of the spleen

The spleen is an important site of erythropoiesis, the clearance of infected RBCs (iRBCs), and immune system activation in response to blood-stage malaria (17). In the present study, the body weight of infected mice was observed to decline following infection with PbK173. The spleen weight of infected groups was observed to increase beginning at 3 dpi. In response to infection, all mice experienced significant splenomegaly, but the splenic index was significantly higher in infected BALB/c mice.
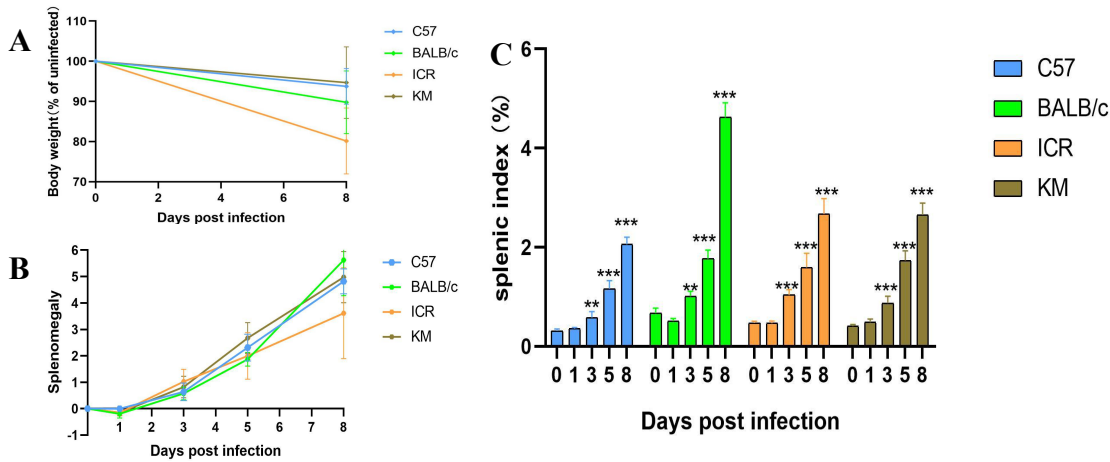

FIG 3: The change in body weight (A) and spleen weight (B) in the four strains of mice, spleen weights were normalized to the weight of uninfected groups (%). C: Splenic index in uninfected and PbK173 infected mice. Spleen weights of infected mice were determined on days 3, 5, and 8 post infection. The splenic index was determined as the ratio of spleen weight to body weight. All data are presented as mean ± SD, n=6. ** and *** indicate statistical significance at $p < 0.01$ and $p < 0.001$ compared with the uninfected group, respectively.
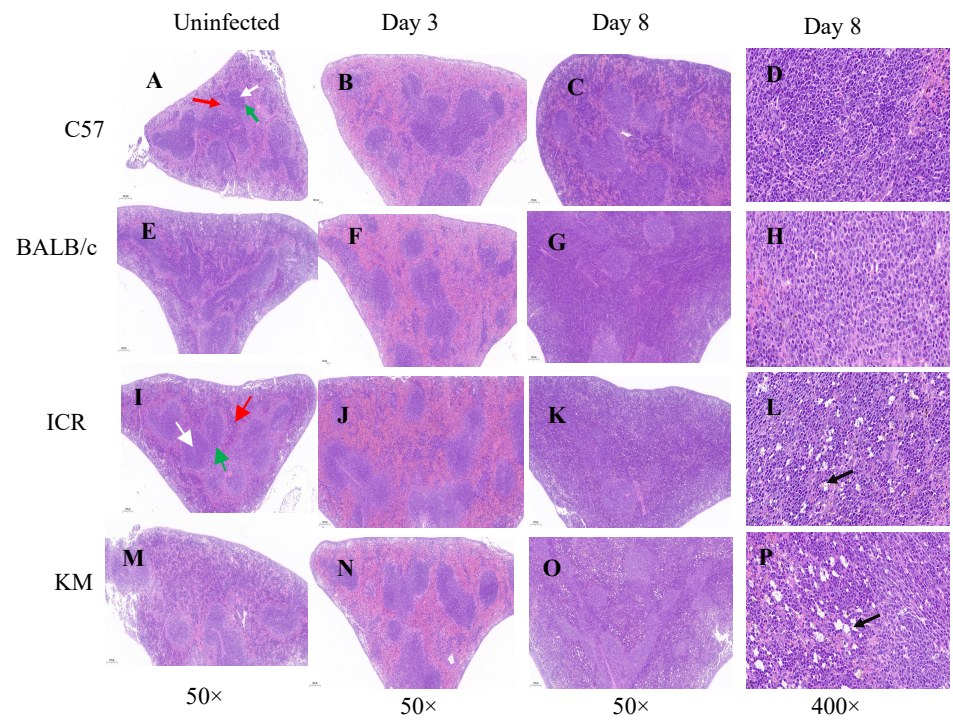
FIG 4: H&E stained sections of spleen from infected or uninfected mice. white arrows: white pulp; red arrows: red pulp; green arrows: marginal zone; black arrows highlight vacuolation.

Figure 4 shows the histopathological sections of the spleen tissue. A clear distinction between the red and white pulp, resting follicles, and marginal zones were evident in the spleen of normal uninfected control mice (Fig. 4: A, E, I, M). Severe congestion and enlarged red pulp was observed in spleens of infected mice at 3 dpi (Fig. 4: B, F, J, N). To a similar all mouse strains examined, increases in red and white pulp cellularity was observed, and the clear marginal zones surrounding follicles became inapparent (except in C57BL/6) at 8 dpi (Fig. 4: C, G, K, O). Furthermore, extensive vacuolation in the red pulp at 8 dpi was observed in spleens from ICR and KM mice (Fig. 4: L, P).
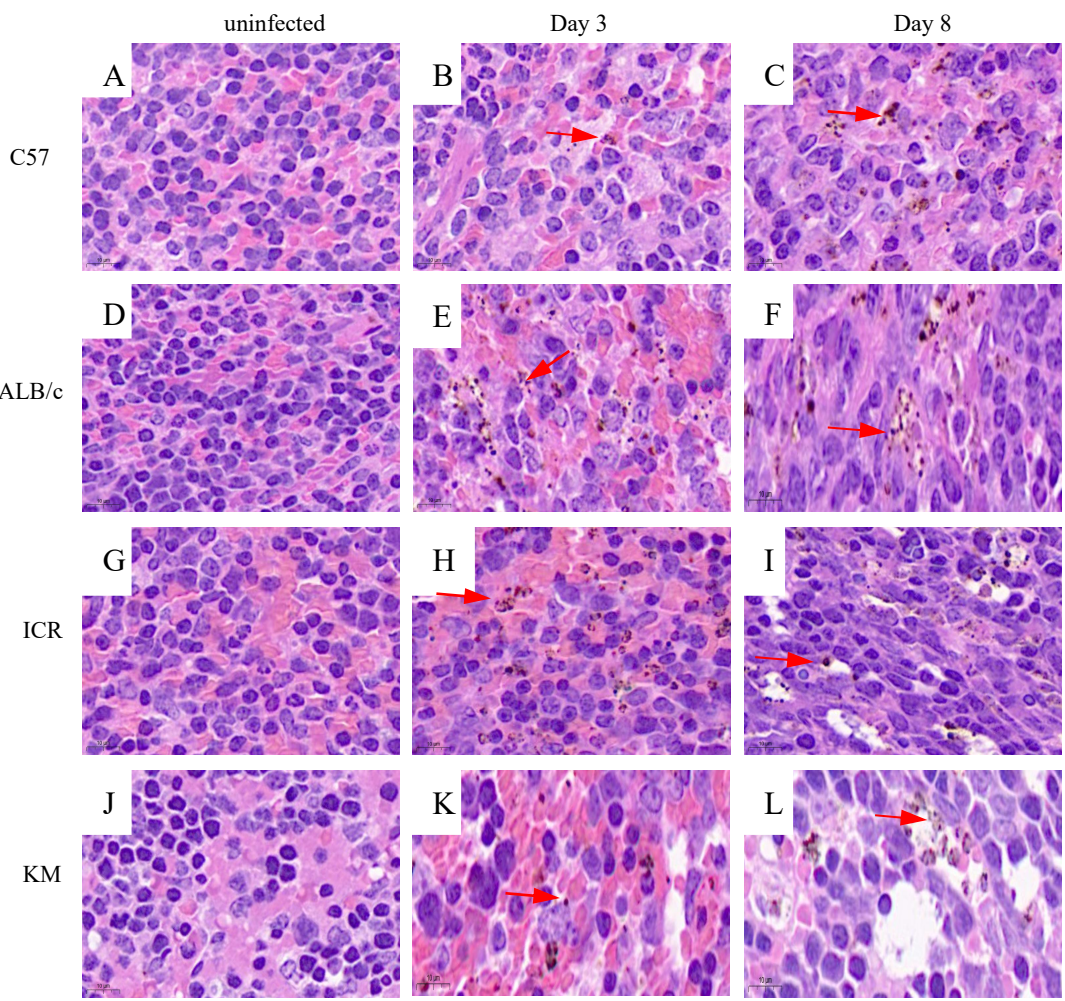
FIG 5: H&E staining of spleen from control and PbK173 infected mice (1000×). Red arrows indicate malarial pigments, which appear as small brown punctate staining.

Spleen sections were prepared and examined via bright-field microscopy coupled with hematoxylin and eosin (H&E) staining (Fig. 5). Parasite pigments in the pulp histiocytes and sinusoidal lining cells were observed at 3 dpi in all PbK173 infected mice. The extent of malaria pigmentation in the spleen is correlated with high parasitemia. The spleens of C57BL/6, BALB/c, ICR, and KM mice filtered late trophozoite stages as well as a fraction of earlier ring-stage parasites out of the blood at 3 dpi (Fig. 5: B, E, H, K). However, KM mice only retained the late trophozoite stage on day 8 (Fig. 5I). This phenomenon may be due to changes of the splenic structure, which could have resulted in alterations to the filtering function of the spleen. Transformations in the red pulp and splenic vasculature may modulate the mechanical retention threshold and regulate the microcirculatory trapping of blood

242   cells in the spleen.
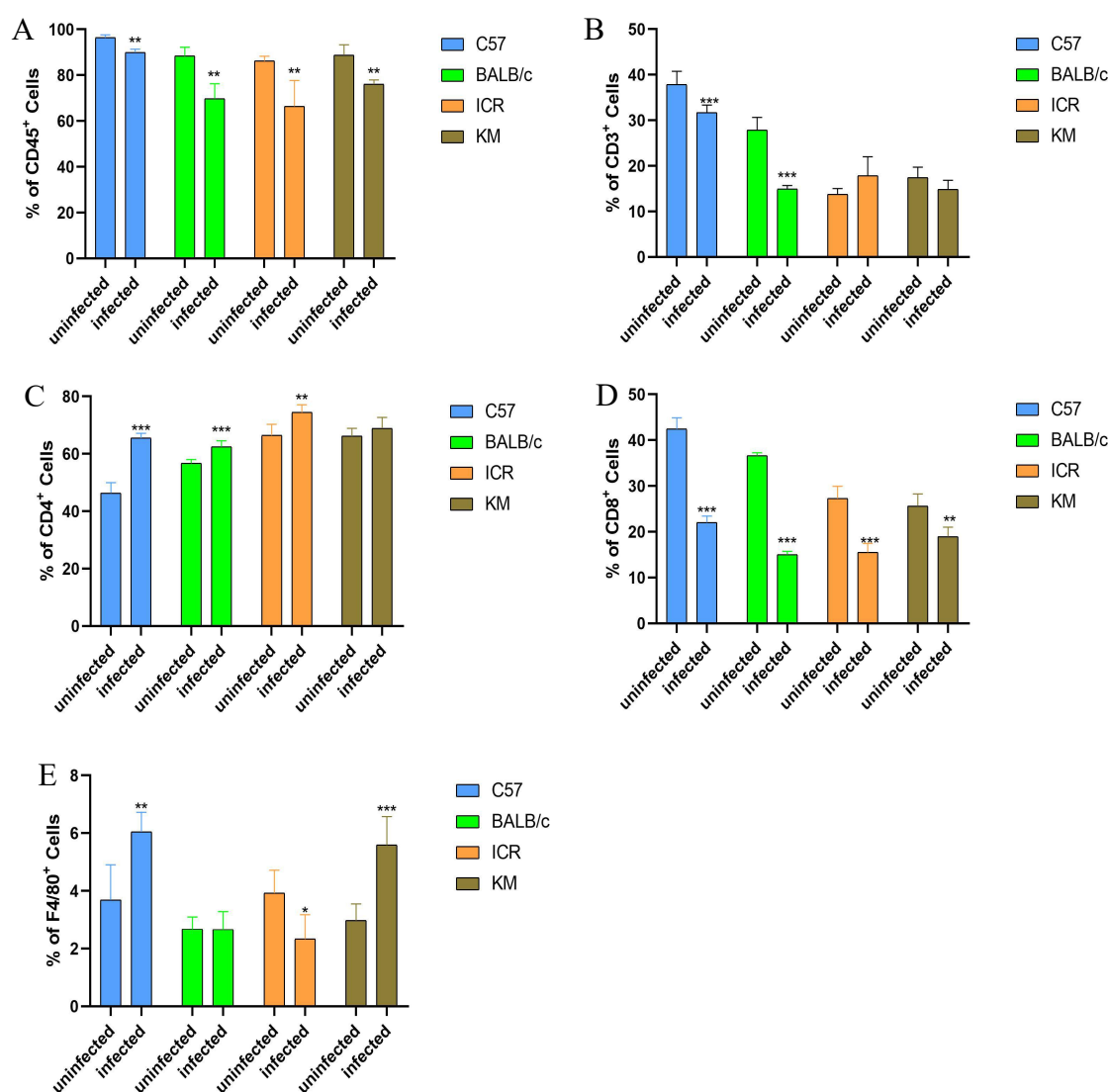
243   **Analysis of Splenocyte Subsets**

FIG 6: Flow cytometry analysis of splenocyte subsets at 8 dpi. Splenocytes of mice infected with parasites were incubated with the required antibodies according to the manufacture's protocol for antibody dilution, incubation duration, etc., and were analyzed in a CytoFLEX flow cytometer. *, ** and *** indicate statistical significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ compared with the uninfected group, respectively.

Next, the distribution of macrophage and T lymphocyte subpopulations were analyzed. Using single-cell suspensions from PbK173 infected or uninfected control spleens, flow cytometry was performed to quantify total leukocytes ($CD45^+$ cells), total T lymphocytes ($CD45^+CD3^+$ cells), T cell subsets ($CD4^+$ and $CD8^+$ cells), and

282 monocytes/macrophages ($CD45^+F4/80^+$ cells) (Fig. 6). A significant decrease in total

283 leukocyte in both C57BL/6, BALB/c, ICR and KM infected mice ($p<0.01$) was

284 observed (Fig. 6A). The data also indicated a significant decrease in the percentages

285 of the T lymphocytes ($CD3^+$ cells, except ICR and KM infected mice) (Fig. 6B) and

286 $CD8^+$ cells (Fig. 6D), while the percentages of $CD4^+$ cells (Fig. 6C) in both C57BL/6,

287 BALB/c, ICR and KM infected mice increased.

288 The trends in macrophage percentages differed between the 4 strains of mice. The

289 percentage of macrophages did not change significantly in BALB/c infected mice. A

290 decrease in the percentage of macrophages was however observed in the infected ICR

291 mice. Conversely, a significant increase in the percentages of the macrophages in

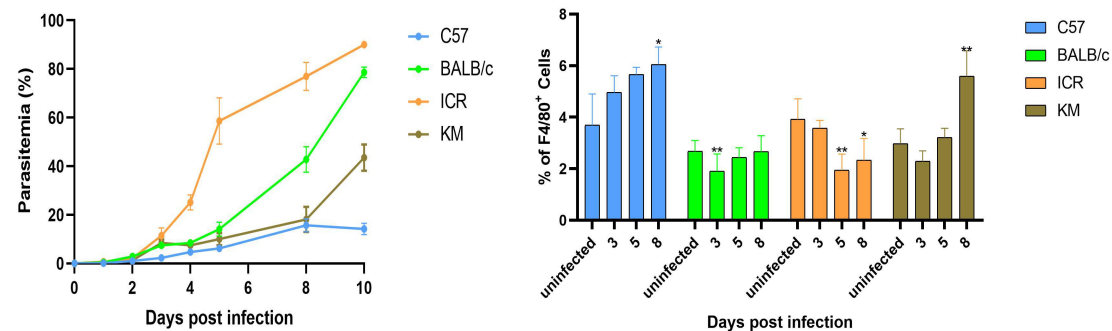292 C57BL/6 and KM infected mice as compared to uninfected mice was observed (Fig.

293 6E).

FIG 7: Parasitemia and flow cytometry analysis of macrophages in C57BL/6, BALB/c, ICR and KM mice over the course of PbK173 infection. * and ** indicate $p < 0.05$, $p < 0.01$ compared with the uninfected group, respectively.

306 Splenic red pulp macrophages, located between the splenic cords and venous

307 sinuses, are well positioned to clear iRBCs and are important for controlling

308 blood-stage malaria (18). In the present study, after infection with PbK173, the

309 percentages of macrophages in the spleen of C57BL/6 mice exhibited the greatest

310 increase of all the mouse strins (Fig. 7B), and the parasitemia progressed the slowest

311 compared to the other mouse strains (Fig. 7A).

312 The percentage of macrophages in the spleen of KM mice was higher than

313 uninfected group on 8 dpi. The spleens in KM mice exhibited structural

314 disorganization and remodeling, which likely affected the mechanical retention

315 threshold. As a result, only late trophozoite stages were retained, which led to a

316 significantly higher parasitemia in KM than C57BL/6 mice. The percentages of

317 macrophages in ICR infected mice were lower than uninfected mice, and the

318 parasitemia was higher than other strains during the course of PbK173 infection (Fig.

319 7).

320 These data show that the rate of splenic mechanical filtration and the splenic

321 macrophages may be important factors in determining an individual's total parasite

322 burden and potentially influencing the pathogenesis of malaria, and different genetic

323 backgrounds of mice have different mechanisms for controlling malaria infection in

324 the spleen.

325

326 **DISCUSSION**

327 In this study, the survival time of mice infected with PbK173 varied, although the

328 infection was uniformly lethal. Mice of the C57BL/6 strain were the most resistant,

329 while ICR mice were the most susceptible. Both BALB/c and KM mice were

330 intermediate.

331 Changes in blood cell counts are characteristic of plasmodium infection.

332 Haematological changes during the course of a malaria infection, such as anaemia,

333 thrombocytopaenia, leukocytosis, and leucopenia are defining. Malaria infections also

334 induce lymphocytopenia, which is accompanied by an increase in neutrophil count,

335 and is a sign of systemic inflammation. Neutrophils (also known as

336 polymorphonuclear cells) are the most common white blood cell in the body (19) and

337 are one of the immune system's first lines of defense against infection. They can

338 neutralize pathogens through several mechanisms, including phagocytosis, the

339 production of reactive oxygen species (ROS) and other antimicrobial products, or by

340 the formation of neutrophil extracellular traps (NETs) (20). Additionally, they also

341 play a role in the activation and regulation of the immune response through the

342 secretion of cytokines and chemokines (21), and the presentation of foreign antigens

343 (22). A substantial body of work has been conducted to investigate the role of

344 neutrophils in malaria. It has been determined that during infection, neutrophils are

345 activated and are capable of clearing malaria parasites by a number of mechanisms.

12

346 In this experiment, the above hematological parameters of different mouse strains

347 exhibited similar trends with respect to changes in the parameters, although the

348 changes in the percentages of monocytes varied. Cells of the monocyte/macrophage

349 lineage are one of the main sources of cytokines in malaria-infected individuals.

350 While some of the cytokines may be of importance for parasite clearance (eg, IL-12)

351 (23), others may be major contributors to disease progression (eg, TNF) (24).

352 Monocytes recognize *Plasmodium falciparum* biological products and Pf-infected

353 erythrocytes directly through pattern recognition receptors (PRR) (25), as well as

354 complement- or IgG-opsonized erythrocytes and parasite components via complement

355 receptors and Fcγ receptors (26). Activated monocytes have several important effector

356 functions in the host defense against malaria, including phagocytosis (27), cytokine

357 production (28), and modulation of adaptive immune responses (29,30). At 5 dpi,

358 compared with the uninfected groups, the percentages of monocytes in BALB/c and

359 C57BL/6 mice were elevated, whereas the values in ICR and KM mice were reduced.

360 Conversely, the parasitemia in ICR mice was significantly higher than KM mice.

361 These results indicate that perhaps the immune cells in peripheral blood are only part

362 of the host response necessary to control plasmodium infection.

363 The spleen is a key site for removal of parasitized red blood cells, generation of

364 immunity and production of new red blood cells during malaria. The importance of

365 the spleen for the control of malaria was confirmed by studying the response of

366 splenectomized humans and rodents to infection. Humans with acute *P. falciparum*

367 malaria who had previously undergone splenectomy had decreased clearance of

368 iRBCs from the circulation (31). The mice subjected to partial splenectomy presented

369 a level of parasites similar to that of non-splenectomized mice, while the animals

370 subjected to full splenectomy had twice the amount of circulating parasites (32).

371 Furthermore, parasite clearance after drug treatment was delayed in splenectomized

372 patients, with RBCs containing dead parasites being retained in the circulation for

373 prolonged periods, compared with individuals with a functional spleen (33).

374 During acute attacks of malaria, splenomegaly is one of the typical signs of malaria,

375 and the degree of splenomegaly often impacts the host's ability to mount a successful

376 response to the parasite (34). Besides an increase in the organ volume and mass, the

377 spleen also exhibits structural disorganization and remodeling. These changes include

378 expansion of the red pulp, transient loss of the marginal zone, increased vasculature,

379 and activation of barrier cells, which may establish a blood-spleen barrier that can

380 drastically alter splenic blood circulation (35,13,36). In this study, the spleen index of

381 infected groups was observed to increase from 3 dpi. Severe congestion and enlarged

382 red pulp was evident in the infected mice. By 8 dpi, infection-induced increases in red

383 and white pulp cellularity and the marginal zones surrounding follicles disappeared in

384 all strains of mice examined. However, the spleens of C57BL/6 and BALB/C infected

385 mice maintain their structural integrity integrity, although the spleen index of

386 BALB/C changed the most. The spleen of ICR and KM mice exhibited severe

387 vacuolation, and the splenic structure was highly atypical, with many of the features

388 absent at this time. This could be a result of the spleen structures of mice with

389 different genetic backgrounds possessing different tolerances and pathologies to

390 infection with malaria.

391   During the erythrocytic stages of malaria infection, the spleen plays a critical role

392 in the host immune response. Elimination of infected erythrocytes occurs through

393 activation of cellular and humoral immune responses, and through mechanical

394 filtration. White and red pulp structures have specific functions in the human spleen.

395 The white pulp is a major control center for the humoral immune response, especially

396 to circulating antigens. The red pulp exerts a unique and subtle control of the surface

397 integrity and biomechanical properties of erythrocytes. To be left in circulation, RBCs

398 must be fit enough to cross a very specific structure of red pulp sinuses, the

399 interendothelial slit (IES). Older erythrocytes, or those modified by innate or acquired

400 conditions, are eventually retained in the splenic red pulp and processed by red pulp

401 macrophages (RPMs) (37).

402   During asexual replication (including the sequential ring, trophozoite, and schizont

403 stages), parasite maturation induces changes in the host RBC with novel proteins

404 synthesis (38,39). As the parasite develops, the infected RBC (iRBC) loses its

405 biconcave shape and progressively becomes spherical and rigid (40). Furthermore, the

406 surface area-to-volume ratio decreases, the shear elastic modulus of the plasma

407 membrane, and the cellular viscosity increase (41). The loss of RBC deformability is

408 not limited to mature stages, but starts soon after parasite invasion. During the ring

409 stage (within the first 16–24 h after RBC invasion), iRBC undergo up to 9.6% surface

410 area loss (42,43). The altered deformability of the plasmodium-infected RBC may

411 result in increased retention in the spleen. More than 50% of ring-iRBC are retained

412 upon *ex vivo* transfusion through human spleens (43). These retention and

413 accumulation processes stem from the splenic screening of RBC deformability (44).

414 However, no direct evidence exists demonstrating the correlation among the rate of

415 splenic mechanical filtration, macrophages, and infection severity. In this study, at 3

416 days post PbK173 infection, malaria pigments were observed in the red pulp in great

417 abundance. The pigments consisted of parasites in the ring and trophozoite stages.

418 In a systemic pathological study of cerebral malaria in African children, enlarged

419 spleens and abundant malaria pigments in splenic macrophages were observed in the

420 majority of the 103 fatal cases. These observations point to an important role of the

421 spleen in parasite control. In this study, the number of macrophages in the spleen of

422 C57BL/6 mice infected with PbK173 was higher than that in uninfected controls, and

423 the parasitemia was lower than other strains during. The percentage of macrophages

424 in the spleen of infected ICR mice was lower than the uninfected group, and the

425 parasitemia increased the fastest. During the infection period, the ratio of

426 macrophages in the spleen of BALB/c mice was not significantly different from that

427 of the uninfected group. The growth rate of the parasitemia was lower than in ICR

428 mice, but higher than that of C57BL/6 mice. This can be explained by the fact that

429 macrophages complement the filtering function of the spleen to control parasitic

430 infections.

431 Artemisinin-based combination therapies (ACTs) are the standard of care to treat

432 uncomplicated falciparum malaria. However, resistance to artemisinins was first

433 reported through observations of a 100-fold reduction in parasite clearance rate in

434 Pailin, Western Cambodia, in 2009 (45). The clearance rate is defined as a parasite

435   clearance half-life $\geq$ 5 hours following treatment with artesunate monotherapy or an

436   ACT (46). Unfortunately, this has become a common issue in Southeast Asia (SEA).

437   Artemisinin resistance has been associated in with multiple nonsynonymous single

438   nucleotide polymorphisms (NS-SNPs) in the propeller domain of the gene encoding

439   the *P. falciparum* K13 protein (K13PD) (47,48). In Africa, significantly prolonged

440   clearance has yet to be observed (49).

441   The delayed clearance phenotype initially observed in SEA does not represent

442   "drug resistance" in the traditional use of the term. Antimicrobial drug resistance is

443   typically defined by quantifiable shifts in the cytostatic or cytocidal potency of the

444   antimicrobial drug. It is important to emphasize that as a 3-day course of artemisinin

445   has never been considered a curative regimen. Artemisinins remain effective, even if

446   they require a longer treatment course or other modifications to the

447   combination-treatment regimen (50). It is not clear if a delay in parasite clearance

448   with artemisinin treatments be defined as drug "resistance."

449   The spleen controls malaria infection by removing the plasmodium from the blood.

450   Splenic functions of mice with different genetic backgrounds vary, and when

451   splenomegaly occurs after malaria infection, the prognosis is not favorable. However,

452   it is not clear if splenomegaly affects the organ's ability to clear parasites from the

453   blood.

454   In summary, the filtering function of the spleen and the expression of macrophages

455   may also be involved in the control of malaria infection. Mice with different genetic

456   backgrounds have different filtering functions in the spleen from the expression of

457   macrophages. The tolerance is also different, which may be the reason for the

458   differential susceptibility of different strains of mice to PbK173.

459

460   **MATERIALS AND METHODS**

461   **Parasite strains and culturing conditions**

462   *Plasmodium berghei* K173, a gift from Dr. Dai of Chengdu University of TCM,

463   was serially passaged *in vivo* in mice. Infected blood was harvested at day 5–7

464   post-infection and stored as frozen stabilates in Alsever's solution containing 10%

465   glycerol.

Mice and infection

467   Male C57BL/6, BALB/C, ICR, and KM wild-type (WT) mice (18~22g,) were used

468   in this study. Animals were purchased from Weitonglihua (Beijing, China). A total of

469   6 mice per group were infected intraperitoneally with 10^7 PbK173-infected RBCs and

470   were provided water and standard laboratory mouse chow diet *ad libitum* throughout

471   the experiment. All mice were housed in pathogen-free animal facilities at the

472   Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

Measurement of hematologic parameters and parasitemia

474   Complete blood counts were obtained with a XN-1000V［B₁］blood analyzer

475   (Sysmex, Japan). Parasitemia (i.e., the percentage of infected RBCs) was assessed at

476   each time point by microscopic counts of thin blood smears stained with Giemsa

477   solution (Sigma -Aldrich, USA).

Isolation of immune cells from mouse spleen

479   Spleen samples were surgically removed and weighed in a sterile hood. One part of

480   each spleen sample was removed and fixed 4% paraformaldehyde for histopathologic

481   examination, and the remainder was used for isolation of splenocytes.

482   Spleens harvested under aseptic conditions were ground into small pieces and

483   passed through a sterilized 200 mesh to prepare crude splenocyte suspensions at room

484   temperature. Samples were then centrifuged at 1000 rpm for 8 min at 4 °C, and the

485   remaining splenocyte suspension was re-suspended in red blood cell Lysis Buffer

486   (Thermo Fisher Scientific, USA). After a 10 min treatment, 1× PBS was added to

487   dilute the samples, and then centrifuged at 1000 rpm for 8 min at 4 °C. The pelleted

488   splenocytes in each group were washed twice and adjusted to concentrations of 5 ×

489   10^6 cells/mL with 1× PBS.

Analysis of Splenocyte Subsets

491   The single cell splenocyte suspensions were stained with the following anti-mouse

492   antibodies: CD45-KO525, CD3-FITC, CD4-PC5.5, CD8-APC, and F4/80-PE

493   (Proteintech Group, USA). Splenocytes were incubated with monoclonal antibodies in

17

494   the dark for 30 min at 4 ℃ . According to the manufacturer's instructions, the

495   specificity of labeling was confirmed by isotype-matched antibody staining controls.

496   The labeled cells were analyzed using a CytoFLEX flow cytometer (Beckman coulter,

497   USA).

Histological examination

499   Spleen tissues were fixed in 4% paraformaldehyde, dehydrated through graded

500   alcohol, embedded in paraffin, sectioned at a thickness of 3 μm, and then stained with

501   hematoxylin & eosin (H&E) and Giemsa solution according to standard procedures.

502   Then, the stained slides were mounted in neutral balsam and covered with coverslips.

503   Histopathologic changes were observed by light microscopy (BX43F Olympus,

504   Japan).

Statistical Analysis

506   Data were analyzed using SPSS 19.0 (IBM,USA) and reported as mean ± SD.

507   Significant differences between groups were analyzed using one-way ANOVA, and

508   are designated as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ relative to the

509   uninfected control groups. Survival curves were calculated using GraphPad Prism 8.0

510   (GraphPad Software,USA).

511

ETHIC STATEMENT

513   Experimental protocols were approved by the Laboratory Animal Ethics Committee

514   of the Institute of Chinese Materia Medica, China Academy of Chinese Medical

515   Sciences (license number SCXK 2016-0006).

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