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Host genetics in malaria: lessons from mouse studies

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Abstract
Malaria remains a deadly parasitic disease caused by *Plasmodium*, claiming almost half a million lives every year. While parasite genetics and biology are often the major targets in many studies, it is becoming more evident that host genetics plays a crucial role in the outcome of the infection. Similarly, *Plasmodium* infections in mice also rely heavily on the genetic background of the mice, and often correlate with observations in human studies, due to their high genetic homology with humans. As such, murine models of malaria are a useful tool for understanding host responses during *Plasmodium* infections, as well as dissecting host-parasite interactions through various genetic manipulation techniques. Reverse genetic approach such as quantitative trait loci studies and random mutagenesis screens have been employed to discover novel host genes that affect malaria susceptibility in mouse models, while other targeted studies utilize mouse models to validate observation from human studies. Herein, we review the findings from the past and present studies on murine models of hepatic and erythrocytic stages of malaria and speculate on how the current mouse models benefit from the recent development in CRISPR/Cas9 gene editing technology.

Introduction

Malaria remains one of deadliest diseases in the world, responsible for approximately half a million deaths in 2015, according to the World Health Organisation (WHO), despite the current global eradication effort. Historically, malaria has been known as a disease of poverty, mostly affecting populations with poor socio-economic status who cannot readily access antimalarial treatments (O'Meara et al. 2009; Yadav et al. 2014). Furthermore, many antimalarials have failed due to the emergence of antimalarial-resistant parasites. Perhaps one of the most notable examples of such resistance development is chloroquine, which was widely used as a monotherapy in the 1940s (Coatney 1963). However, since the 1950s, resistance to chloroquine has been reported in Thailand (Payne 1987), and it spread rapidly to Africa by the 1970s, rendering chloroquine unsuitable as a monotherapy for malaria (Wellems and Plowe 2001). Similarly, resistance has been reported for all subsequent commercially available antimalarials, including sulfadoxine-pyrimethamine (Cowman et al. 1988), mefloquine (Nosten et al. 2000) and even the current first-line antimalarial treatment—the artemisinin derivatives (Dondorp et al. 2009; Noedl et al. 2008). On the other hand, vaccine development for malaria is facing significant challenges due to the complexity of the parasite lifecycle and wide antigenic variation (Scherf et al. 2008), with the sole commercially available malaria vaccine, RTS,S, only providing short-lasting protection of 30–50% in human population (Alonso et al. 2004; Mian-McCarthy et al. 2012). Taking these factors into consideration, there is an urgent need to develop an effective control for malaria infections, either preventative or curative.

Remarkably, the co-evolutionary relationship between malaria parasites and humans over thousands of years has resulted in strong selective pressure on both the host and the parasites. Malaria is caused by *Plasmodium* parasites, of which there are currently five major species of *Plasmodium* that infect humans—*Plasmodium falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi. P. falciparum*, the most widespread and lethal species, which is responsible for the highest morbidity and mortality associated with malaria, while *P. vivax* is also widespread but has a lower mortality rate. *Plasmodium* has a complex lifecycle within the human host (reviewed in Cowman et al. 2016). The parasites, in the form of sporozoites, are injected into the human host during

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a blood meal by female *Anopheles* mosquitoes. They travel to the liver via the bloodstream infecting the hepatocytes and remaining clinically asymptomatic. Each sporozoite differentiates into thousands of merozoites, which upon egress, invade the red blood cells (RBCs) and begin to replicate asexually from ring to schizont stage before egressing to invade more RBCs. This erythrocytic stage of *Plasmodium* lifecycle is responsible for the clinical symptoms and complications of malaria, including severe anemia, fever, cerebral malaria, coma, and death. Some merozoites develop into gametocytes, which are taken up by the mosquitoes during a blood meal, initiating the sexual stage in mosquitoes.

During the asexual stage in the human host, the parasites have evolved strategies to evade detection by the host immune system. Notably, *Plasmodium* exhibits substantial genetic variation, such as RBC binding proteins (reviewed in Cowman and Crabb 2006), which contributes significantly to the invasion process into RBCs. In addition, mature blood-stage parasites often express genes encoding virulence factors, allowing them to adhere to the endothelium to avoid exposure to splenic macrophages (reviewed in Crabb and Cowman 2002). As such, the *Plasmodium* parasites remain the prime target of malaria research in attempts to discover parasite targets for the development of effective vaccines or treatments. However, it is also important to note that the host also plays a significant, if not equal role during malaria infections. In fact, there is accumulating evidence on the role of host genetics affecting the outcome of malaria infections, particularly certain genetic disorders such as sickle cell disease and thalassemia (Nagel 1990). As such, it is crucial to gain a better understanding of the genetic basis underlying host-parasite interactions, particularly using murine models, as we develop new therapies for malaria.

Mice have been used extensively for malaria research since the late 1940s (Stephens et al. 2012), following the discovery of rodent parasites, allowing us to manipulate both host and parasite genetics in an in vivo settings. The four laboratory species of rodent parasites commonly used to model human parasites are *P. chabaudi, P. berghei, P. yoelii*, and *P. vinckei*, with each species displaying substantial similarities to key characteristics of human parasites. For instance, *P. chabaudi* exhibit similarities to the erythrocytic stage of *P. falciparum*, where it invades both mature RBCs and reticulocytes (Carter and Walliker 1975); however, unlike *P. falciparum*, it does not sequester in the brain. *P. berghei*, on the other hand, primarily infects reticulocytes, similar to *P. vivax*, and it is usually used as a model for cerebral malaria (Craig et al. 2012). *P. yoelii* sporozoites have high hepatocyte infectivity and cause similar immunological response as the pre-erythrocytic stage of *P. falciparum* (Weiss 1990), thus making it an attractive species to model liver-stage infections. Coupled with the high genetic homology between mice and humans, murine malaria infections provide an accurate model for studying host-parasite interaction in malaria.

Therefore, to gain a better perspective of our current understanding of the host genetics in malaria infections, here we reviewed the findings and the established knowledge from various mouse studies on hepatic and erythrocytic stage malaria (summarized in Table 1). There is an extensive body of literature covering both innate and adaptive immune responses towards malaria infection, as well as cerebral malaria, and are not discussed in detail here as they fall outside the scope of this review. We will, however, present the different roles and examine how different genes affect the pathogenesis of murine malaria, and compare these to the findings from human studies.

Genetic linkage studies in mouse models

Similar to humans, mice exhibits different susceptibility to malaria infection depending on their genetic background, as demonstrated by several studies on inbred strains of laboratory mice (Bopp et al. 2010; Foote et al. 1997; Laroque et al. 2012; Stevenson et al. 1982). Notably, mouse strains such as C57BL/6 and DBA/2J were regarded as resistant to *P. chabaudi* infections, whereas BALB/c, C3H/HeJ, SJL/J, and SM/J strains were susceptible (Laroque et al. 2012; Stevenson et al. 1982). Similar studies were also performed on *P. berghei* infections, where SJL/J, C57BL/6, and C3H/HeJ strains were susceptible, while DBA/2J and AKR/J strains were resistant with improved survival (Bopp et al. 2010). Such major differences prompted several quantitative trait loci (QTL) studies to identify the gene loci responsible for increased resistance or susceptibility against several murine *Plasmodium* species, including *P. chabaudi, P. berghei*, and *P. yoelii*.

So far, a total of ten QTL have been described for resistance against *P. chabaudi* infections (known as *Chabaudi* resistance loci or *Char*). Using susceptible C3H/He and SJL/J and resistant C57BL/6 inbred mouse strains, *Char1* and *Char2* were the first loci to be isolated, which localized to the distal region of chromosome 9 and the central region of chromosome 8, respectively (Foote et al. 1997; Fortin et al. 1997). Both loci contributed to the survival and peak parasitemia of *P. chabaudi adami* DS-infected mice. Interestingly, *Char1* has also been implicated to confer resistance to *P. yoelii* 17XL infection (designated as *P. yoelii malaria resistance, Pymr*) (Ohno et al. 2001), and more recently, *P. berghei* (Bopp et al. 2013), suggesting an essential role in providing protection against multiple species of malarial parasites. Further studies might yield potentially attractive targets to combat malarial infections. Several candidate genes have been proposed, including haptoglobin (*Hp*), erythrocyte antigen 1 (*Ea1*), transferrin (*Trf*),

Table 1 Mouse genes and gene loci controlling susceptibility towards rodent malaria identified from various studies

Genetic mutations or loci	Chromosomal location [candidate genes]	Resistance/susceptible phenotypes	References
Gene loci identified in QTL studies			
Char1 (or *Pymr)*	Proximal Chr 9 [*Hp, Ea1, Trf, Rbp1, Rbp2*]	*P. chabaudi adami* DS • Reduced parasitemia • Increased survival *P. yoelii* 17XL • Reduced parasitemia • Increased survival *P. berghei* ANKA • Increased survival	Bopp et al. (2013), Foote et al. (1997), Ohno et al. (2001)
Char2	Proximal and distal Chr 8 [*GypA, iL15, Msr1, Cspg3, Il12rb1*]	*P. chabaudi adami* DS • Reduced parasitemia • Increased survival *P. chabaudi chabaudi* AS • Reduced parasitemia • Increased survival *P. chabaudi chabaudi* 54X • Altered parasite growth rate	Burt et al. (2002), Foote et al. (1997), Fortin et al. (1997), Hernandez-Valladares et al. (2004a), Lin et al. (2006)
Char3	Chr 17 [*Tnf, Lta, Ltb, Tnfsf, Ifi15, Thy2*]	*P. chabaudi adami* DS • Reduced parasitemia • Increased survival *P. chabaudi chabaudi* 54X • Altered parasite growth rate	Burt et al. (1999), Hernandez-Valladares et al. (2004a)
Char4	Chr 3 [*Pklr*]	*P. chabaudi chabaudi* AS • Reduced parasitemia • Increased survival • Early parasite clearance • Increased erythropoiesis	Min-Oo et al. (2003)
Char5 *Char6*	Chr 5 [*Ache, Epo, Hspb1, Cora1, Ncf1, Act1*]	*P. chabaudi chabaudi* 54X • Altered parasite growth rate	Hernandez-Valladares et al. (2004a)
Char7	Chr 17 [*C3, Il25, Ir5*]	*P. chabaudi chabaudi* 54X • Altered parasite growth rate	Hernandez-Valladares et al. (2004a)
Char8	Chr 11 [*Il3, Il4, Il5, Il12b, Il13, Csf2*]	*P. chabaudi chabaudi* 54X • Affects later stage parasitemia	Hernandez-Valladares et al. (2004b)
Char9	Chr 10 [*Vnn1, Vnn3*]	*P. chabaudi chabaudi* AS • Reduced parasitemia	Min-Oo et al. (2007a)
Char10	Chr 9 [*Dapk2, Ireb2, Splq4*]	*P. chabaudi chabaudi* AS • Increased survival, but normal parasitemia	Laroque et al. (2017), Min-Oo et al. (2010b)
Belr	Chr 17 [*Trem2*]	*P. berghei* ANKA • Reduced liver-stage expansion	Goncalves et al. (2008), Goncalves et al. (2013)

Table 1 (continued)

Genetic mutations or loci	Chromosomal location [candidate genes]	Resistance/susceptible phenotypes	References
Genes identified from genome-wide mutagenesis screens			
Ank-1	Chr 8	*P. chabaudi adami* DS • Increased survival • Reduced parasitemia • Reduced RBC invasion, and intra-erythrocytic maturation • Increased RBC bystander clearance	Greth et al. (2012), Huang et al. (2017), Huang et al. (2016), Rank et al. (2009)
Tfrc	Chr 16	*P. chabaudi adami* DS • Increased parasitemia *P. yoelii* NL • No difference	Lelliott et al. (2015)
Sptb	Chr 12	*P. chabaudi adami DS* • Increased survival • Reduced parasitemia • Reduced RBC invasion, and intra-erythrocytic maturation • Increased RBC bystander clearance *P. yoelii* NL • Increased survival • Reduced parasitemia	Lelliott et al. (2017)
Ampd3	Chr 7	*P. chabaudi adami* DS • Increased survival • Reduced parasitemia • Increased RBC clearance • Increased erythropoiesis	Hortle et al. (2016)
Genes investigated in targeted studies			
Human *HbS*	Transgenic expression	*P. berghei* ANKA • Increased survival • Reduced cerebral malaria incidence • Increased splenic clearance *P. yoelii* 17XL • Increased survival • Reduced parasitemia	Hood et al. (1996), Shear et al. (1993)
Heme oxygenase-1 (*Hmox1*)	Chr 8	*P. berghei* ANKA • Protection against cerebral malaria • Increased liver load (sporozoites) *P. chabaudi chabaudi* • Gene knockout causes reduced survival and succumbs to liver failure *P. yoelii* sporozoites • Gene knockout reduces liver load	Epiphanio et al. (2008), Ferreira et al. (2011), Pamplona et al. (2007), Seixas et al. (2009)

Table 1 (continued)

Genetic mutations or loci	Chromosomal location [candidate genes]	Resistance/susceptible phenotypes	References
Alpha-tocopherol transfer protein (*Ttpa*)	Chr 4	*P. berghei* NK65 • Gene knockout increases mouse survival, lowers parasitemia, increases DNA damage to parasites *P. berghei* ANKA • Gene knockout protects against cerebral malaria *P. yoelii* 17XL • Gene knockout increases mouse survival and lowers parasitemia	Herbas et al. (2010a), Herbas et al. (2010b)
Band 3 (*Slc4a1*)	Chr 11	*P. yoelii* 17XL • Gene knockout reduces parasitemia	Baldwin et al. (2015)
Glycophorin C (*Gypc*)	Chr 18	*P. berghei* ANKA • Gene knockout reduces merozoite invasion into RBCs	Yiangou et al. (2016)
Piezo1	Chr 8	*P. berghei* ANKA • Gain-of-function mutation lowers parasitemia and protects against cerebral malaria	Ma et al. (2017)
Fech	Chr 18	*P. chabaudi adami* DS • Increased survival • Reduced parasitemia	Smith et al. (2015)
Cd36	Chr 5	*P. yoelii* 17XNL • Gene knockout increases parasitemia, reduces survival *P. berghei* ANKA • Gene knockout reduces tissue sequestration	Franke-Fayard et al. (2005), Thylur et al. (2017)
Icam1	Chr 9	*P. berghei* ANKA • Deficiency increases survival, reduces sequestration in lung *P. chabaudi chabaudi* AS • Deficiency increases blood parasitemia, reduces sequestration in spleen and liver	Cunningham et al. (2017), Li et al. (2003)
Cd81	Chr 8	*P. yoelii* 265BY sporozoites • Gene knockout reduces hepatocyte invasion *P. berghei* NK65 sporozoite • No difference in hepatocyte invasion	Silvie et al. (2003)
Class B, type I scavenger receptor (SR-BI) (*Scarb1*)	Chr 5	*P. berghei* ANKA sporozoites • Deficiency reduces hepatocyte invasion *P. yoelii* 265BY sporozoites • Deficiency reduces hepatocyte invasion	Yalaoui et al. (2008)

and retinol-binding proteins (*Rbp1* and *Rbp2*) (Foote et al. 1997); however, the causative gene(s) underlying the resistance to *P. chabaudi* of *Char1* still remains to be identified.

Char2 was first discovered along with *Char1*, and was later refined in attempts to identify the causative genes using techniques including advanced intercross lines (AIL) approach between susceptible A/J and resistant C57BL/6 mice (Hernandez-Valladares et al. 2004a), and congenic lines from susceptible C3H/He and resistant C57BL/6 mice (Burt et al. 2002). These studies suggested that *Char2* was the result of additivity of two adjacent loci—the original proximal region (Foote et al. 1997; Fortin et al. 1997; Hernandez-Valladares et al. 2004a), and the distal region (Burt et al. 2002; Lin et al. 2006), indicating two loci were involved in the resistance phenotype. The original studies proposed glycophorin A (*GypA*), interleukin 15 (*Il15*), and macrophage scavenger receptor 1 (*Msr1*) to be potential candidate genes (Foote et al. 1997; Fortin et al. 1997), while the AIL study further suggested chondroitin sulfate proteoglycan 3 (*Cspg3*) and interleukin-12 receptor beta1 (*Il12rb1*) to be possible candidate genes. However, no studies were conducted to further refine *Char2* and map it to the single gene level.

Char3 was initially localized to the proximal end of chromosome 17, and appeared to be involved in the clearance of *P. chabaudi adami* DS parasites through immune response, although at later time compared to *Char1* and *Char2* (Burt et al. 1999). Although originally mapped to the mouse major histocompatibility locus, the H2 complex, refinement of *Char3* was carried out using (A/J x C57BL/6J) F11 AIL mouse population (Hernandez-Valladares et al. 2004a). As such, other candidate genes were proposed, including tumor necrosis factor alpha (*Tnf*), beta (*Lta*) and c (*Ltb*), tumor necrosis factor ligand (*Tnfsf*) superfamily members, interferon activated gene 15 (*Ifi15*), and thymus cell antigen 2 (*Thy2*) (Hernandez-Valladares et al. 2004a). In the same study, refinement of *Char3* revealed another locus, *Char7*, which is about 10 cM apart and exerts an additive effect with *Char3* (Hernandez-Valladares et al. 2004a). The authors suggested complement component 3 (*C3*), interleukin-25 (*Il25*), and immune response 5 (*Ir5*) to be the candidate genes for *Char7*, and also discovered another pair of linked loci with epistatic effects, *Char5* and *Char6*, mapped on chromosome 5, which appeared to affect parasitemia. The authors suggested several candidate genes for these loci, including acetylcholinesterase (*Ache*), erythropoietin (*Epo*), heat shock 27-kDa protein 1 (*Hspb1*), correlation in cytokine production 1 (*Cora1*), NADPH oxidase subunit (*Ncf1*), and the actin-related gene 1 *(Act1)*(Hernandez-Valladares et al. 2004a).

The same authors later discovered *Char8*, mapped to chromosome 11, using the same AIL cross between (A/J x C57BL/6J) F11 mouse population. The locus *Char8* appeared to act in the later stage of malaria infection and its neighboring region corresponds to the human chromosome 5q31–33, which contains many candidate genes encoding immunological effectors (Hernandez-Valladares et al. 2004b). These effectors have been repeatedly implicated in many linkage studies influencing parasitemia in human populations (Milet et al. 2010; Naka et al. 2009; Rihet et al. 1998). Notable candidate genes for *Char8* include but are not limited to interleukin-3, -4, -5, -12b, and -13, granulocyte–macrophage colony-stimulating factor 2 (*Csf2*) (Hernandez-Valladares et al. 2004b). Interestingly, both *Il3* and *Csf2* have been shown to confer resistance to *P. chabaudi* infections (Auclair et al. 2014; Riopel et al. 2001), indicating that they might be the likely candidates for *Char8*.

An alternate approach to QTL studies involves the use of recombinant congenic strains (RCSs) from C57BL/6J and A/J inbred strains to study quantitative traits and dissect the genetic factors affecting *P. chabaudi* infections (Fortin et al. 2001b), by crossing deviant and susceptible strains. Through these lines, *Char4* was mapped on chromosome 3, which affects the parasitemia levels and survival rate (Fortin et al. 2001a). The positional candidate gene approach revealed an amino acid substitution I90N of pyruvate kinase (*Pklr*) gene resulting in a non-functional protein. This mutation is associated with splenomegaly and compensatory erythropoiesis, which is thought to confer *P. chabaudi* resistance (Min-Oo et al. 2003). Remarkably, pyruvate kinase (PK) deficiency is also associated with malaria resistance in humans (Ayi et al. 2008; Durand and Coetzer 2008), which further validates the findings from QTL mapping studies in mouse models. The resistance mechanisms of pyruvate kinase deficiency in humans (Ayi et al. 2008) appear to be consistent with the mouse studies (Min-Oo et al. 2004). Pyruvate kinase deficiency causes hemolytic anemia in both human and mice, which results in elevated erythropoiesis and shorter RBC lifespan, limiting the replication of blood-stage parasites in mature RBCs (Ayi et al. 2008; Min-Oo et al. 2004). Furthermore, the protective effect of *Pklr* gene in both human and mice is correlated with the severity of the mutations (Min-Oo et al. 2007b; van Bruggen et al. 2015), suggesting a dose-dependent relationship which could be investigated further in mouse models.

The same approach was used to identify *Char9* on the proximal region of chromosome 10, which also affects the peak parasitemia during *P. chabaudi* infections (Min-Oo et al. 2007a). The authors performed positional cloning of *Char9* and proposed that pantetheinase-encoding genes vanin-1 and 3 (*Vnn1* and *Vnn3*) to be the likely candidate genes due to their tissue-specific gene expressions during malaria infection between resistant and susceptible mouse strains. This is further supported by a more recent investigation on mice with low pantetheinase activity which revealed an increased susceptibility towards cerebral

malaria and possibly increased peak parasitemia (Rommelaere et al. 2015). Low pantetheinase activity was found to correlate with high RBC oxidative stress and shorter half-life, indicating a protective effect conferred by serum pantetheinase activity (Rommelaere et al. 2015). An alternative explanation involves cysteamine, a product of pantetheinase activity, which has been shown to impair the replication of intra-erythrocytic *P. chabaudi* in vivo and *P. falciparum* in vitro (Min-Oo et al. 2010a), and possibly protect against cerebral malaria caused by *P. berghei* (Penet et al. 2008).

Char10 was later discovered using RCS panel used to identify *Char4*, where the mouse strain AcB62 exhibited susceptibility towards *P. chabaudi* infections despite carrying the protective *Char4* allele ($Pklr^{I90N}$) (Min-Oo et al. 2010b). AcB62 was crossed with another PK deficient mouse strain CBA/Pk carrying $Pklr^{G338D}$, and linkage analysis was carried out on the F2 generation. *Char10* locus on chromosome 9 was found to exhibit significant linkage with malaria susceptibility in the context of PK deficiency (Min-Oo et al. 2010b). A recent follow-up study was conducted which revealed the crucial role of *Char10* regulating the PK-specific resistance towards blood-stage malaria, where mice carrying *Char10* exhibit less splenomegaly and a less pronounced erythropoietic response during malaria infections, despite the presence of $Pklr^{G338D}$ mutation (Laroque et al. 2017). Although *Char10* appeared to be the modifier for PK-related malaria resistance, the candidate genes for *Char10* remains to be investigated. Candidate genes for *Char10* are likely to be involved in erythropoiesis, such as death-associated protein kinase 2 (*Dapk2*) and iron responsive element binding protein 2 (*Ireb2*) (Min-Oo et al. 2010b).

In contrast, limited QTL studies have been conducted on the liver stage of *Plasmodium* infection. Goncalves et al. (2008) conducted genetic mapping on congenic mouse strains between *P. berghei*-susceptible C57BL/6 and resistant BALB/c strains, and discovered *Belr1* locus on chromosome 17, which appeared to confer resistance towards liver-stage infection of *P. berghei*. Interestingly, *Belr1* locus was shown to co-localizes with *Char7*, which were mapped distally to the H2 complex (Goncalves et al. 2008). Although in the follow-up study, the authors proposed triggering receptor expressed on myeloid cells 2 (TREM2) to be the causative gene underlying *Belr1* (Goncalves et al. 2013), and a role for TREM2 in the liver-stage infection was clearly demonstrated using mice deficient for this gene. However, it is unknown if the resistance mediated by *Char7* and *Belr1* could involve the same genetic factors controlling both the blood and liver stage of murine *Plasmodium* infections, as both were localized distally of H2 locus of chromosome 17, with *Char7* in between 55 and 66 Mb and *Belr1* in between 37 and 65 Mb.

Genome-wide mutagenesis screens in mouse models

Apart from QTL studies, another forward genetic approach involves using random mutagenesis of the mouse genome, followed by phenotypic screening for resistance against *Plasmodium* infections. *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen has been utilized to discover novel genetic factors controlling susceptibility to *Plasmodium* infections (Bauer et al. 2015), particularly against experimental cerebral malaria (Bongfen et al. 2012; Torre et al. 2015) and blood-stage malaria (Greth et al. 2012; Rank et al. 2009). ENU mutagenesis screens for blood-stage malaria typically involve monitoring changes to peak parasitemia during infection, with a secondary hematological screen as a useful indicator of the potential candidate ENU-induced genetic mutations since RBCs play a major role in the survival of blood-stage malaria parasites. Exome sequencing of mice with ENU-induced mutations allows the identification of the causative genetic mutations, which may be studied in further detail.

In the past decade, ankyrin-1 (*Ank1*) has been described in several ENU studies as a gene of interest influencing blood parasitemia (Greth et al. 2012; Huang et al. 2017, 2016; Rank et al. 2009). *Ank1* is an important component of RBC cytoskeletal structure responsible for anchoring the RBC cytoskeletons to the RBC membrane, and ankyrin-1 mutations are associated with hereditary spherocytosis (HS) in human populations (Eber et al. 1996; Facer 1995; Savvides et al. 1993). Mice carrying ENU-induced *Ank1* mutations were consistently found to have lower blood parasitemia in all these studies, suggesting a disrupted RBC cytoskeleton is detrimental to *P. chabaudi* survival. This observation was recently confirmed on ENU mutations disrupting Spectrin-beta (*Sptb*) gene on the RBC cytoskeleton (Lelliott et el. 2017). These observations are consistent with in vitro studies, where human erythrocytes with *ANK1* mutations are resistant to *P. falciparum* invasion and impair intra-erythrocytic growth of the parasites (Facer 1995; Schulman et al. 1990). Interestingly, recent studies of multiple *Ank1* mutations in mouse models revealed the allelic heterogeneity of *Ank1* during malaria infections (Huang et al. 2017). *Ank1* mutations could impair parasite invasion, intra-erythrocytic growth and parasite clearance depending on the nature and the location of mutations within *Ank1* gene (Huang et al. 2017, 2016). Despite the apparently strong evidence from mouse studies, the association of HS with malaria resistance in human population still remains to be further clarified, as the majority of reported HS cases affect populations in non-endemic regions (Gallagher 2005; Yawata et al. 2000). Nevertheless, these

mouse studies have provided important clues for dissecting the relationship between RBC membrane proteins and *Plasmodium* parasites.

ENU mutagenesis screening also revealed transferrin receptor 1 (*Tfrc*) gene to be involved in determining malaria susceptibility (Lelliott et al. 2015). Transferrin receptors are crucial for haem synthesis during erythropoiesis, and deficiency causes reduced iron levels in erythroblasts and subsequently microcytosis (Levy et al. 1999). Low bioavailability of iron in the host body is often associated with increased resistance towards *Plasmodium*, as observed with a reduced risk of malaria in children with iron deficiency anemia (IDA) (Gwamaka et al. 2012; Jonker et al. 2012), while iron supplementation increases malaria risk (Clark et al. 2014; Goheen et al. 2016). Interestingly, the *Tfrc* mutation described by Lelliott et al. (2015) exhibit normal hemoglobin levels and increased malaria susceptibility despite low levels of transferrin receptors, suggesting a different mechanism in play compared to IDA. Furthermore, IDA in human populations is often the result of malnutrition (Miller 2013), rather than genetic mutations, therefore, these observations are not directly comparable. Nevertheless, mouse models with *Tfrc* mutations provide an excellent platform to further investigate the relationship between iron levels and *Plasmodium* in a controlled environment, which is unachievable with human populations.

Mice carrying mutations in the adenosine monophosphate deaminase (*Ampd3*) gene was discovered, during an ENU mutagenesis screen, to be highly resistant to *P. chabaudi* infections (Hortle et al. 2016). *Ampd3* is involved in maintaining ATP levels in various tissues, and is usually supressed in RBCs, to prevent ATP loss, which would otherwise lead to oxidative damage (Mahnke and Sabina 2005; Tavazzi et al. 2000). The authors described a mutation $Ampd3^{T689A}$, which produces a constitutively active AMPD3, causes ATP loss and a significantly shortened RBC half-life, limiting the replication of blood-stage parasites (Hortle et al. 2016). Therefore, the observed increased malaria resistance is due to the secondary effect of ATP depletion promoting eryptosis, rather than a direct effect on *Plasmodium* growth. While no human studies have associated impaired host purine metabolism with malaria resistance, the resulting increased oxidative stress is a common malaria resistance mechanism shared by several well-studied genetic disorders, including G6PD deficiency, sickle cell anemia, thalassemia and PK deficiency (Ayi et al. 2009; Cytlak et al. 2013; Ibrahim et al. 2014; Peters and Van Noorden 2009). This study also opened up new possibilities of controlling blood-stage *Plasmodium* infection via oxidative damage through targeting purine metabolism pathways.

Targeted gene studies in mouse models

Reverse genetics approaches have also been employed to investigate the genetic factors underlying malaria resistance in mouse models. High homology between human and mouse genome (Emes et al. 2003) enables scientists to translate observations between human and mouse studies. As such, mouse models are often used for validating hypotheses from human or in vitro studies through gene knockout or knock-in mouse models, in particular genes affecting cerebral malaria (de Oliveira et al. 2014; Kassa et al. 2016) and liver- and blood-stage malaria. Some notable studies are discussed as below, particularly those involving RBCs, parasite cytoadherence and oxidative stress.

The blood-stage *Plasmodium* parasite relies heavily on the optimal RBC microenvironment to support its growth and replication. Therefore, changes to RBC properties, including hemoglobin, membrane proteins, or cytoskeleton, are expected to significantly impair parasite survival. Mouse models provide an exceptional platform to further investigate the resistance mechanisms behind these genetic disorders. For instance, sickle cell trait in human populations has long been associated with resistance and improved host survival during *Plasmodium* infection (Allison 1954; Beutler et al. 1955; Billo et al. 2012). Many in vitro studies so far have demonstrated that reduced parasite invasion and growth (Friedman 1978; LaMonte et al. 2012) and cytoadherence (Cholera et al. 2008) and rosette formation (Carlson et al. 1994) are the mechanisms of protection. On the other hand, mouse models of sickle cell disease, which are also resistant to various species of murine malaria (Hood et al. 1996; Shear et al. 1993), have revealed additional mechanisms that could explain malaria resistance through splenic clearance (Shear et al. 1993) and the induction of heme oxygenase (Ferreira et al. 2011). Mice with functional heme oxygenase were thought to be protected against malaria infection by counteracting the cytotoxic effect of the released oxidative free heme, protecting the host from cerebral and non-cerebral forms of malaria (Pamplona et al. 2007; Seixas et al. 2009). Interestingly, heme oxygenase appeared to exacerbate the liver stage of malaria infection in the mouse models (Epiphanio et al. 2008), by blocking the inflammatory responses (Rodrigues et al. 2008), thus providing a favorable environment for the establishment of liver infection. These findings suggest a mixed role for heme oxygenase plays during malaria infections.

Mouse studies have also proposed alpha-tocopherol transfer protein (TTPA) to be protective against malaria infections. TTPA is responsible for regulating the levels of vitamin E in blood plasma (Arita et al. 1997), which is

thought to act as an anti-oxidant to scavenge free radicals (Levander et al. 1995). TTPA knockout mice exhibited significantly reduced parasitemia and increased survival during malaria infections, with an observed increase in oxidative damage to the parasites (Herbas et al. 2010b), and also protected against cerebral malaria (Herbas et al. 2010a). This protective effect was demonstrated to be directly due to reduced vitamin E levels, highlighting the importance of antioxidants for *Plasmodium* parasite survival. This study also opened up the prospect of improving the current malaria treatments with drugs controlling levels of antioxidants, which has been further investigated in subsequent studies (Herbas et al. 2015; Kume et al. 2016). The mice with treated with probucol, which reduces plasma TTPA concentration, showed increased protection against *P. yoelii* infection and enhance the effect of dihydroartemisinin-mediated killing of parasites (Herbas et al. 2015; Kume et al. 2016).

Apart from those described in ENU mutagenesis screen, other RBC cytoskeletal and membrane proteins have also been topics of interest in malaria research due to their roles in parasite invasion and intra-erythrocytic growth. Of relevance here are the glycophorins and band 3, which have been repeatedly validated to be associated with malaria resistance in human studies (Facer 1983; Jarolim et al. 1991; Maier et al. 2003). Similarly, band 3 null mice with glycophorin A and protein 4.2 deficiencies exhibited significant resistance to *P. yoelii* invasion, and it is currently thought that this protein complex is required for the attachment of the sporozoites to the RBC surface (Baldwin et al. 2015). Another recent study on erythrocytes differentiated from mouse embryonic stem cells with glycophorin C deletion, and normal band 3, were resistant to *P. berghei* invasion (Yiangou et al. 2016), suggesting glycophorin C is essential for *P. berghei* invasion, but not band 3. It is likely that various murine parasite species exhibit differences in terms of their RBC invasion pathways, similar to human parasites (Iyer et al. 2007). Nevertheless, both in vitro and in vivo models provide important evidence and clues to the possible invasion pathways of malaria parasites in a human host.

A more recent study on Piezo1, a mechanosensitive ion channel of RBCs (Zarychanski et al. 2012), has revealed a potential role of this gene in malaria resistance (Ma et al. 2017). *Piezo1* mutations are associated with hereditary xerocytosis (HX) in humans, and manifests as RBC dehydration as a result of certain gain-of-function mutations in *Piezo1* (Albuisson et al. 2013; Bae et al. 2013). The authors generated mice carrying a *Piezo1* mutation corresponding to human mutation R2456H. These mice exhibited a HX-like phenotype, as well as increased resistance to *P. berghei* infections, as evidenced by a slower increase in parasitemia and protection from cerebral malaria (Ma et al. 2017). These observations led to the use of comparative genomics approach to identify Piezo1 alleles in populations in malaria endemic regions. Remarkably, blood from African populations carrying *PIEZO1* E756del mutation have been associated with increased resistance to *P. falciparum* infection in vitro (Ma et al. 2017), indicating a potential role for Piezo1 to confer resistance during malaria infections.

The presence or absence of certain enzymes in erythrocytes could also play a role in determining parasite survival, such as the well-established glucose-6-phosphate dehydrogenase (G6PD) deficiency and PK deficiency (Durand and Coetzer 2008; Miller et al. 1984). Despite being present at a residual level, heme biosynthetic enzymes have also been recently implicated to affect parasite intra-erythrocytic growth, where parasites were thought to scavenge these enzymes for their own heme biosynthesis (Ke et al. 2014; Nagaraj et al. 2013). In particular, the presence of host ferrochelatase (FECH), a rate-limiting enzyme in heme biosynthesis, is required for optimal parasite growth (Smith et al. 2015). Mice with low ferrochelatase activity as the result of *Fech* mutations exhibited increased survival and lower parasitemia during *P. chabaudi* infection (Smith et al. 2015). This observation was further supported by in vitro studies on erythrocytes from human patients with erythropoietic protoporphyria, as well as RBCs treated with ferrochelatase inhibitor, in which *P. falciparum* growth is reduced (Smith et al. 2015). It is likely that other heme biosynthetic enzymes might have similar effects, although no further studies have been done using mouse models.

In addition to RBC properties, endothelial receptors, such as CD36 and ICAM-1, have been linked with the virulence of *Plasmodium* parasites to cause sequestration in various organs and cerebral malaria (Favre et al. 1999; Omi et al. 2003; Serghides et al. 2003). CD36 has another role in mediating immune response during malaria infection (Erdman et al. 2009; Stewart et al. 2010), and mouse models with CD36 knocked-out have increased parasitemia with low survival rate, and reduced pro-inflammatory cytokines, Th1 and phagocytic responses during *P. yoelii* infections (Thylur et al. 2017). Another study, however, reported that CD36 knockout mice exhibited reduced sequestration in lung and adipose tissues during *P. berghei* infections (Franke-Fayard et al. 2005), thus providing crucial evidence for the dual role of CD36 during malaria infections. Similarly, ICAM-1 is thought to be crucial for cytoadherence of *P. falciparum* (Turner et al. 1994) and the ICAM-1 deficient mice displayed reduced sequestration in lung tissues and increased survival during *P. berghei* infections, despite high level of pro-inflammatory cytokines such as TNF-α (Li et al. 2003). A more recent study on blood-stage *P. chabaudi* infections also revealed similar findings, where ICAM-1 knockout mice exhibit higher blood parasitemia, but reduced parasite sequestration in spleen and liver (Cunningham et al. 2017), whereas ICAM-1 overexpression led to increased liver load

(Medeiros et al. 2013). ICAM-1 is also an important molecule for the adhesion of host leukocytes in the liver during malaria infections (McNamara et al. 2017), implicating a protective role for ICAM-1 to locate and kill liver-stage parasites, which further suggests an opposing role for ICAM-1 during malaria infections.

In terms of liver-stage malaria, host factors involved in lipoprotein trafficking, such as class B, type I scavenger receptor (SR-BI), liver-fatty acid binding protein (L-FABP), and CD81, have been associated with sporozoite invasion. Both CD81 and SR-BI are found on the hepatocyte surface, and were thought to be necessary for other pathogens, including hepatitis C virus (Vercauteren et al. 2012). CD81 knockout mice are resistant to *P. yoelii* sporozoite invasion, consistent with the observation that CD81-deficient human hepatocytes were resistant to *P. falciparum* sporozoite invasion in vitro (Silvie et al. 2003). Interestingly, CD81-deficient hepatocytes did not impair the invasion of *P. berghei* (Silvie et al. 2003), possibly indicating an alternative invasion pathway employed by *P. berghei* sporozoites. On the other hand, SR-BI is required for *P. berghei* and *P. falciparum* sporozoite invasion in vitro (Yalaoui et al. 2008), and hepatocyte invasion is correlated with SR-BI levels in in vivo siRNA silencing experiment, where expression of SR-BI is not abrogated (Rodrigues et al. 2008). However, the same could not be said for SR-BI knockout mice. Rodrigues et al. (2008) observed a similar infection levels in SR-BI knockout mice compared to wild-type mice, and the authors proposed that the high levels of heme oxygenase level (Van Eck et al. 2007) in these mice favored parasite growth, possibly counteracting the resistance from SR-BI deficiency. Yalaoui et al. (2008), however, reported increased resistance to *P. yoelii* sporozoite invasion for SR-BI knockout mice. Although the exact mechanisms remain elusive, it is thought that SR-BI causes conformational changes to CD81, allowing the sporozoite to invade (Yalaoui et al. 2008). SR-BI also activates L-FABP (Yalaoui et al. 2008), which has been shown to interact with a parasite protein essential for liver-stage survival, upregulated in sporozoites-3 (UIS3) (Mikolajczak et al. 2007). SR-BI knockout mice exhibited reduced levels of L-FABP, presenting a possible resistance mechanism for these mice.

Conclusion and future perspective

Taking these studies together, it is evident that mouse models are invaluable resources for investigating host factors involved in malaria susceptibility. High homology between human and mouse genetics allows smooth translation of many findings from mouse studies to humans, which not only provides insight to host-parasite interactions, but also identifies potential druggable host targets to be used as malaria treatment. Nevertheless, murine *Plasmodium* are not perfect models for human *Plasmodium*, in which specific murine models are required to investigate particular aspect of human *Plasmodium* pathogenesis (Badell et al. 1995; Stephens et al. 2012; Wykes and Good 2009). Moreover, observations in mouse models do not always reflect those in human studies, as evidenced by various failed clinical vaccine trials despite showing promises in mouse models (Crompton et al. 2010).

Despite these limitations, reverse genetic strategy shows the power to identify novel biology and host genes that were not implicated previously in malaria susceptibility and were not enriched in GWAS studies. Whereas powerful, the generation of ENU mutagenized mice is time consuming and expensive. QTL mapping using F2, RIL or RCS enables an unbiased approach to determine the mechanisms underlying host resistance to malaria infection but lack sufficient mapping resolution to rapidly identify alleles responsible for these traits. To overcome these limitations, new models for malaria research are being developed, and their usages are on the rise. One of these is the humanized mouse models, which is thought to create a more clinically relevant *P. falciparum* model, while still retain the benefits of an in vivo model (Siu and Ploss 2015). However, immunodeficient mice are required to ensure a successful xenograft, suggesting certain immunological aspect of *Plasmodium* infection cannot be examined in this model. Alternatively, Collaborative Cross (CC) mouse lines or Diversity outbred panel (DO), rather than individual inbred strains, could be employed to further dissect the genetic basis of malaria resistance. CC or DO are a panel of mouse strains or heterogenous stocks derived from eight inbred strains, which is thought to capture up to 90% genetic variation of the mouse population (Roberts et al. 2007), allowing highly accurate modeling to replicate the level of genetic variation in human populations. The availability of high-resolution isogenic maps from CC or DO would also greatly simplify QTL studies, and also allowing a more realistic model for examining host-pathogen interactions (Churchill et al. 2004; Threadgill and Churchill 2012). For example, CC mouse lines have been used for QTL studies to investigate susceptibility to various genetic disorders (Atamni et al. 2017; Donoghue et al. 2017) and infectious diseases (Durrant et al. 2011; Ferris et al. 2013; Vered et al. 2014), highlighting the benefits CC and DO brings onto the table to complement the conventional mouse lines for QTL studies.

In addition, a highly efficient genetic manipulation technique such as CRISPR-Cas9 technology has shown promises to generate highly specific mutations in mice (Lelliott et al. 2017; Chen et al. 2016; Qin et al. 2015; Yang et al. 2014). Not only could this technique improve the current reverse genetics approach on mouse models, it could also open up new possibilities to investigate host genetics in other

systems, including in vitro hematopoietic stem cells (Gundry et al. 2016) and mosquitoes (Hammond et al. 2016). Two recent studies show the power of CRISPR/Cas9 gene editing technology in mouse to investigate host resistance to malaria infection. The first one aimed to specifically disrupt the binding interaction between Ankyrin1 and Spectrin-beta by the generation of a single point mutation in Sptb using CRISPR/Cas9 in mice. The edited mice were protected against severe *P. chabaudi* infection (Lelliott et al. 2017). A second recent study has employed CRISPR-Cas9 technology to generate a calcium transporting ATPase (*Atp2b4*) knockout in mice (Lessard et al. 2017), which has been implicated in severe malaria resistance from GWAS study (Timmann et al. 2012). This new exciting development will enable to investigate other malaria susceptibility genes using CRISPR/Cas9 technology and validate GWAS hits for malaria susceptibility in mice.

Altogether, it is apparent that mouse models will remain one of the most important models for malaria research for the foreseeable future. The emergence of these new models and techniques at our disposal would greatly benefit the investigation into the relationship between host genetics and malaria parasites in mouse models, potentially enabling the development of a personalized malaria vaccine or treatment to ensure effective malaria control.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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