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Of men in mice: the success and promise of humanized mouse models for human malaria parasite infections

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Abstract

Forty percent of people worldwide are at risk of malaria infection, and despite control efforts it remains the most deadly parasitic disease. Unfortunately, rapid discovery and development of new interventions for malaria are hindered by the lack of small animal models that support the complex life cycles of the main parasite species infecting humans. Such tools must accommodate human parasite tropism for human tissue. Mouse models with human tissue developed to date have already enhanced our knowledge of human parasites, and are useful tools for assessing anti-parasitic interventions. Although these systems are imperfect, their continued refinement will likely broaden their utility. Some of the malaria parasite's interactions with human hepatocytes and human erythrocytes can already be modeled with available humanized mouse systems. However, interactions with other relevant human tissues such as the skin and immune system, as well as most transitions between life cycle stages *in vivo* will require refinement of existing humanized mouse models. Here, we review the recent successes achieved in modeling human malaria parasite biology in humanized mice, and discuss how these models have potential to become an valuable part of the toolbox used for understanding the biology of, and development of interventions to, malaria.

Introduction

Malaria presents a significant global health burden, with 300–500 million clinical cases and approximately 800,000 deaths caused by *Plasmodium falciparum* and *Plasmodium vivax* (Kappe *et al.*, 2010). Due to limitations in the current laboratory models of human malaria parasites, much of what is known about *Plasmodium* biology has been extrapolated from rodent-infecting malaria parasites such as *Plasmodium berghei* and *Plasmodium yoelii* (Lindner *et al.*, 2012). After transmission by an infected *Anopheles* mosquito, the sporozoite stage traverses skin cells invades skin capillaries, is taken up by the blood stream and transported to the liver. Once there, it traverses the sinusoidal endothelium (Mota *et al.*, 2001, Ishino *et al.*, 2004, Tavares *et al.*, 2013), and each parasite establishes itself in a single hepatocyte. In most *Plasmodium* species, all parasites then rapidly replicate as liver

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schizonts and ultimately spawn tens of thousands of exo-erythrocytic merozoites. Upon release from hepatocytes, parasites enter the bloodstream, invade red blood cells, and initiate intra-erythrocytic replication, which causes disease. However, in *Plasmodium vivax* infection, a subset of parasites form dormant liver stages, called 'hypnozoites'. These parasites are thought to reactivate at a later time, and again cause blood stage infections. These clinical episodes associated with recurrent blood stage infection are known as "relapse". During blood stage infection, some parasites develop into sexual forms called gametocytes, which are transmitted to the mosquito vector and complete the life cycle (Figure 1).

The complex life cycle of human malaria parasites and the specificity towards human cell infection have long constituted barriers to study many aspects of these parasite's biology. Here we review humanized mouse models that have already facilitated the study of *P. falciparum* liver stages and blood stages *in vivo*. We aim to highlight where previous models have been unable to fully capture the complex biology of human-infecting malaria parasites and how humanized mouse models will provide opportunities for physiologically relevant studies in the future.

Shared features of humanized mouse models

The humanized mouse models discussed here utilize a common set of mutations and genetically deficient backgrounds for immuno-compromised status that facilitate engraftment with human cells and tissues. The Severe Combined Immune Deficiency (SCID) mouse (Bosma *et al.*, 1983) carries a point mutation in the PRKDC kinase that eliminates B and T cells (Blunt *et al.*, 1995, Kirchgessner *et al.*, 1995). The Non-Obese Diabetic (NOD) mouse dramatically reduces clearance of human hematopoetic cells (Takenaka *et al.*, 2007), in part because of a polymorphism in the signal regulator protein alpha (SIRPα) gene that enhances binding to human CD47 and diminishes macrophage phagocytosis (O'Brien *et al.*, 2002). Natural killer cells also have limited activity in NOD mice because of a defect in the NKG2D receptor (Ogasawara *et al.*, 2003). These properties have made the NOD background preferred for the development of models that utilize xenotransplantation of hematopoetic cells.

Targeted genes deletions have also contributed to the immuno-compromised status that is required for the development of humanized mouse models. The Recombination activating gene 2 (Rag2) is responsible for VD(J) recombination (Oettinger *et al.*, 1990), and thus its elimination in mice leads to lack of B and T cells (Shinkai *et al.*, 1992). Similarly, deleting the IL-2 receptor gamma chain (IL2Rγ) depletes the common receptor of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, eliminating functional T cells and NK cells. Crosses between these mice have resulted in the NOD/SCID mouse, the NSG mouse (NOD/SCID/IL2Rγ^{null}), the NOG mouse (NOD/SCID/IL2R$\gamma^{truncated}$)(Hasegawa *et al.*, 2011)), and the NRG (NOD/Rag2/IL2Rγ) mouse, each of which will be discussed in the sections that follow (Figure 2).

Modeling human malaria transmission from mosquito vector to host

During *Plasmodium* journey from the salivary glands of the mosquito to the mammalian hepatocyte within the liver parenchyma, sporozoites encounter a diverse landscape of cells

types and tissues. Gliding motility allows *Plasmodium spp.* to travel through the skin (Menard *et al.*, 2013), and cell traversal allows them to pass through cells of any type (Mota *et al.*, 2001). Initially, it was assumed that the parasites' biology in the skin is not species-specific. More recently it has been demonstrated that *P. berghei* can undergo pre-erythrocytic development within skin cells and form exoerythrocytic merozoites (Gueirard *et al.*, 2010) but this complete development does not occur in *P. yoelii* (Voza *et al.*, 2012). These data prompts consideration of whether this so-called 'skin-stage' exists for human *Plasmodium* parasites. If relevant to human infection, the skin stage could impact the drug development strategies for human malaria, as pharmacological interventions developed towards pre-erythrocytic stages in the liver might not affect skin stages. Novel approaches such as humanized mice with bioengineered skin (reviewed in (Carretero *et al.*, 2011)) could be used to understand what role, if any, the skin plays in pre-erythrocytic development of human malaria parasites. The common SCID/NOD background of human-skin-engrafted mouse models, liver and blood models (see next sections) suggests that the 'skin stage' could be incorporated into a comprehensive humanized mouse model of human malaria parasite life cycles.

Modeling human malaria parasite liver stages

The liver stage (LS) infection begins when a *Plasmodium* sporozoite enters a hepatocyte and surrounds itself with the Parasitophorous Vacuole Membrane (PVM). Since liver stage infection is asymptomatic and lasts seven to ten or more days for human *Plasmodium* species, it constitutes an attractive target to prevent progression to disease-causing blood stage infection and further transmission of the parasite.

Limitations of rodent malaria parasite models

Plasmodium species infecting rodents and humans are highly divergent. While some critical factors for pre-erythrocytic infection are known in rodent-infecting *P. berghei* and *P. yoelii*, it remains largely unknown how relevant they are for *P. falciparum* and *P. vivax* pre-erythrocytic infection. For example, the hepatocyte surface protein CD81, which is required for hepatocyte infection by one rodent malaria parasite species appears also necessary for *P. falciparum* infection of hepatocytes (Silvie *et al.*, 2003). However, the receptor tyrosine kinase c-Met appears to be involved in early liver infection by *P. berghei* (Carrolo *et al.*, 2003), but not is not at all important for *P. yoelii* or *P. falciparum* (Kaushansky et al., 2011). Binding to heparan sulfate proteoglycans on the hepatocyte surface facilitates rodent malaria sporozoite invasion (Frevert *et al.*, 1993, Coppi *et al.*, 2007) but whether they facilitate human parasite invasion remains unclear. Interestingly, while rodent malaria parasites infect (Hollingdale *et al.*, 1983, Silvie *et al.*, 2003) and sometimes complete LS development in human hepatocytes (reviewed in (Prudencio *et al.*, 2011)), mouse hepatocytes are unsuitable for both *P. falciparum* and *P. vivax* LS development. Thus, experimentation that directly assesses factors that impact human malaria parasite LS development is critical.

A better understanding of the receptors required for sporozoite invasion of host cells would uncover a remaining mystery about the malaria parasite and could facilitate the development of novel, immuno-competent, humanized mouse models. A parallel approach has been successful in developing a partially humanized model that supports hepatitis C virus (HCV)

infection. More specifically, knock-in mice that express human CD81 and occludin (OCLN) have been used for modeling *in vivo* HCV infection in fully immuno-competent mice (Ploss *et al.*, 2009). The possibility that some of the HCV receptors (i.e. CD81) overlap with those utilized by *Plasmodium spp.* suggests that mice developed for supporting infection with other pathogens could be useful for studying human parasite LS.

Current humanized liver mouse models for human *Plasmodium* pre-erythrocytic infection

The establishment of mouse models that allow for repopulation of mouse livers with human hepatocytes requires two major components: (1) an immuno-compromised recipient mouse to prevent the rejection of human tissue (discussed above), and (2) the initiation of liver injury that depletes mouse hepatocytes and thus creates a niche to allow human hepatocytes to colonize the mouse liver. To date, there are four major approaches that have proven successful in generating liver injury suitable for the establishment of human-hepatocyte repopulated mouse livers: (1) the expression of urokinase Plasminogen Activator (uPA) toxin under the albumin promoter (Rhim *et al.*, 1994); (2) the Fumarylacetoacetate Hydrolase (FAH) knockout that leads to the toxic build-up of fumarylacetoacetate, an intermediate of tyrosine metabolism (Azuma *et al.*, 2007); (3) the expression of the herpes simplex virus type 1 thymidine kinase (HSVtk) transgene (Hasegawa *et al.*, 2011); and (4) the inducible activation of apoptosis via Caspase 8 oligomerization (Washburn *et al.*, 2011). In each of these models the basic premise is similar in that liver injury caused by the death of mouse hepatocytes creates a niche that can be colonized by injected primary human (adult or fetal) hepatocytes. While the uPA model is most established, the advantage of other models is the inducible nature of the liver injury.

The first success in assessing *P. falciparum* liver stage development in liver-humanized mice used Alb-uPA on a SCID background (Mercer *et al.*, 2001). The liver of these mice can be robustly repopulated with human hepatocytes soon after birth and these hepatocytes are susceptible to *P. falciparum* infection (Sacci *et al.*, 2006, Mikolajczak *et al.*, 2011). To our knowledge the model has yet to be used to model *P. vivax* liver stages.

More recently, it has been demonstrated that *P. falciparum* can infect other humanized mouse models. Specifically, FRG KO mice that lack FAH, Rag2, and IL2rγ can be efficiently transplanted with human hepatocytes (FRG KO huHep) (Azuma *et al.*, 2007). We have recently shown that this model supports robust *P. falciparum* LS infection and that it is able to support complete maturation of LS parasites (Vaughan *et al.*, 2012) (Figure 3). When these mice are back-crossed on the NOD background (FRG-NOD-HuHep), the resulting new model can support the transition of exo-erythrocytic merozoites stage to blood stage infection (Vaughan *et al.*, 2012). Encouragingly, atypical small arrested LS that are observed during *in vitro* hepatocyte infection with *P. falciparum* sporozoites (March *et al.*, 2013) are never observed in this model (Vaughan *et al.*, 2012). This fact will be useful moving forward to the study of *P. vivax* liver stage infection, which unlike *P. falciparum* harbors small dormant hypnozoite forms. Thus, models which do not harbor artificially small forms will be critical for understanding *P. vivax* liver stage dormancy (Vaughan *et al.*, 2012).

Thus, to date, only SCID-Alb-uPA and FRG-HuHep mice have been shown to be suitable for the development of *P. falciparum* liver stages. However, other currently existing models have the potential to provide a purely *in vivo* system to study *Plasmodium* biology and pathogenesis. One of these models is created when the HSVtk transgene is expressed within the liver NOG (NOD/SCID/IL2$\gamma^{truncated}$) background mice (TK-NOG), inducing death in mouse hepatocytes and thus allowing for repopulation with human hepatocytes. Because of the NOD and immuno-compromised background, it is possible these mice will be suitable for a combined model of liver and blood stage infection. This model has been demonstrated to be suitable for HCV infection, raising the possibility that it may also be useful for modeling *Plasmodium* liver stage (Kosaka *et al.*, 2013).

Transition from humanized liver infection to human red blood cell infection

At the end of LS growth and replication, exo-erythrocytic merozoites form and exit the host hepatocyte as merosomes, or 'packages' of merozoites surrounded by host plasma membrane (Sturm *et al.*, 2006). Initially this was demonstrated using rodent malaria parasites, but recent work using FRG huHep has shown that *P. falciparum* has a similar exit strategy (Vaughan *et al.*, 2012). Importantly, exo-erythrocytic merozoites released form the liver in FRG-NOD HuHep mice are infectious to human red blood cells (hRBCs) injected 6–7 days after sporozoite infection (Vaughan *et al.*, 2012).

Asexual blood stage replication of human parasites in mice with human RBCs

Blood stage parasites are responsible for all symptoms of malaria infection. A small animal model for *P. falciparum* blood stages would enhance understanding of disease pathophysiology, and is critical for modeling the complete life cycle in small animal models. Complex interactions between infected red blood cells (iRBCs) and other cell types (for example, through the phenomenon of cytoadhesion) can be studied only in a limited capacity through *in vitro* models (Tripathi et al., 2006, Avril et al., 2012). Differences in the cytoadhesion properties of rodent malaria parasites and human malaria parasites have so far prevented the development of accurate *in vivo* model for severe, cerebral (Nacer *et al.*, 2012) and placental malaria (reviewed in (Held *et al.*, 2013)).

Currently available models: advantages and challenges

Several immuno-deficient mouse strains with a humanized RBC compartment have been evaluated as human malaria blood stage models with variable degrees of success (reviewed in (Khan *et al.*, 2012)). In these models, severe immune deficiency has been a prerequisite to support blood stage infection, in large part due to the requirement for hRBC xenotransplatation. The most successful model of *P. falciparum* blood stage infection to date has been the NSG mouse model that lacks functional T, B, NKT and NK cells (Arnold *et al.*, 2011). In this model, remaining elements of the immune system (i.e. macrophages) can be depleted by injection of liposomal-clodronate formulations (Arnold *et al.*, 2010, Arnold *et al.*, 2011). Human red blood cells are then injected at regular intervals to replenish cleared

cells. Reproducible, high parasitemia can be achieved in this model (Arnold *et al.*, 2010, Arnold *et al.*, 2011).

The alternative approach of adding human cytokines to immuno-deficient mouse models has enhanced their capacity to support engraftment of human hematopoetic lineage cells. Human cytokines can be expressed either as knock-in constructs, administered as purified protein, or expressed as transgenes in the liver by hydrodynamic injection of DNA. Expression of human thrombopoietin (TPO) enhances hematopoetic stem cell (HSC) engraftment as well as the differentiation of the common erythroid/megakaryocytic progenitor cells (Rongvaux *et al.*, 2011). Treatment with erythropoietin (EPO) and IL-3 after macrophage depletion and CD34+ cell transplantation in the NSG model (Hu *et al.*, 2011) allows increased hRBC repopulation in the bloodstream. Similarly, phagocytosis of human cells can be minimized by expression of human SIRPα (Strowig *et al.*, 2011). In addition to enhancing maintenance of *P. falciparum* blood stages, models that include immature hRBC by differentiating them from hHSCs ($CD34^+$ cells) might be more suitable for the study of *P. vivax*, as these models develop reticulocytes, which are required for *P. vivax* blood stage infection.

Sexual stages and blood-to-mosquito transmission

High *P. falciparum* parasitemia and relatively stable levels of hRBC chimerism in NSG mice allows for the transition to sexual stages, although mature gametocytes are rarely detected (Arnold *et al.*, 2011), perhaps partially due to the fact that it takes two weeks for *P. falciparum* gametocytes to mature. Interestingly, while asexual stages of *P. vivax* are more challenging to culture, sexual stages might be more straightforward to develop *in vivo* since gametocytes appear to develop more quickly (reviewed in (Galinski *et al.*, 2013). FRG-NOD HuHep mice infected with *P. falciparum* sporozoites allow transition to blood stages, which retain their capacity to become fertile gametocytes and transition to mosquito-infectious forms *in vitro* (Vaughan *et al.*, 2012), paving the way for following the human parasite through multiple transmission cycles in vivo in the laboratory. Transmission blocking interventions are currently only assessed *in vitro*, which does not account for factors such as drug metabolism or gametocyte sequestration that might complicate intervention efforts.

Future Directions for the use of humanized mouse models

Genetic crosses

An immediate application of humanized liver mouse models that allow transition of the liver stage infection to blood stage infection is that such models could support forward genetics research with *P. falciparum*. Genetic crosses have been the workhorse of the geneticist attempting to map genetic determinants of phenotypic variation in animals and plants. Unfortunately human malaria parasite genetic crosses to map determinants encoding phenotypes of importance such as antimalarial drug resistance have only been possible by conducting experimental infections of chimpanzees (Su *et al.*, 2007) with sporozoites derived from mated gametocytes of two distinct parasite strains (e.g. chloroquine resistant versus chloroquine sensitive strains (Su *et al.*, 1997)).

Completing the life cycle

A combined humanized mouse model that can harbor liver stages, allows transitions to blood stages and continuously supports blood stage infection would constitute a major advance. The AFC8-hu HSC/Hep model provides the first step towards such a combined model of hepatocyte and hematopoetic humanization. In this model, AFC8 mice are treated with FK506 binding protein (FKBP), which induces caspase-8 activation and elicits apoptosis in hepatocytes (Washburn *et al.*, 2011). Mice are then transplanted with hepatic progenitor cells and hematopoetic stem cells resulting in hepatocyte (15%–25%) and hematopoietic-derived cell repopulation (>90%) (Bility *et al.*, 2012). It remains unclear if human *Plasmodium* parasites can infect this model, but it can be successfully infected by HCV (Washburn *et al.*, 2011). In a separate model, TK-NOG mice can be transplanted with human HSCs (Hasegawa *et al.*, 2011), and repopulated with human hepatocytes (discussed above), which might also support a combined liver stage-blood stage model.

Repopulating animals with induced pluripotent stem cell-derived cells (iPS cells) could also contribute to a combined liver stage/blood stage model. In this approach, human somatic cells are de-differentiated into stem cells (Takahashi *et al.*, 2006), and then re-differentiated into HSCs (Lengerke *et al.*, 2009) and hepatocytes (Chen *et al.*, 2012). Although this approach has not yet been used in the development of humanized mouse models, the possibility of creating personalized, humanized mice would facilitate the testing of important biological and clinically relevant hypotheses concerning the genetic basis of susceptibility to malaria infection and disease.

Blood stage cytoadherence

One of the most disease-relevant features of *P. falciparum* iRBCs is their ability to cytoadhere to the vascular endothelium. The specific interactions involved are not conserved in rodent malaria species/mouse infections, making them of little use to study this phenomenon. Several receptors on human endothelial cells have been shown to support interactions with hRBCs infected with *P. falciparum*, including thrombospondin (Li *et al.*, 2011), CD36 (Ockenhouse *et al.*, 1988), ICAM-1 (Ockenhouse *et al.*, 1991), and EPCR (Turner *et al.*, 2013). A mouse model that supports human blood stage parasite replication and that expresses humanized receptors on endothelial cells would provide a powerful *in vivo* model of infected hRBC sequestration and its role in malaria pathogenesis.

Models of human immunity

Studies in mice and humans have revealed that both humoral and cellular responses play a role in the development of protective immunity against malaria. It has recently been demonstrated that the SCID-Alb-uPA and FRG mouse models can assess antibody-mediated protection against *P. falciparum* sporozoite challenge by passive transfer of antibodies to mice prior to challenge (Foquet *et al.*, 2013, Sack *et al.*, 2013). To our knowledge, cellular responses have yet to be assessed in any of these models.

Developing a platform that models human immune responses to malaria infection and vaccination remains a challenge. Towards this goal, the expression of human cytokines such as IL-3 and GM-CSF have enhanced human alveolar macrophage production (Willinger *et*

al., 2011), and mice that express M-CSF have enhanced monocyte and macrophage production after HSC transplantation. Human T cells can be generated by repopulating NSG mice with both HSCs and human fetal thymus and liver tissue (Jaiswal *et al.*, 2012) or alternatively through the expression of HLA class I heavy chain and light chain (Shultz *et al.*, 2010). Finally, mice which produce human antibodies through the expression of human immunoglobulins have been developed (reviewed in (Seung *et al.*, 2013)). Mice with these human immune components can be combined with human liver and blood models and would be invaluable in assessing the protective efficacy of immune responses generated by vaccination with candidate *P. falciparum* antigens or to assess the immune responses generated by *Plasmodium* infection.

Use of humanized mouse models to study *Plasmodium vivax*

P. vivax is the most widespread cause of malaria worldwide (Guerra *et al.*, 2010). No long-term propagation systems for blood stages of *P. vivax* exist, in large part because *P. vivax* infection is restricted to reticulocytes (Galinski *et al.*, 2013). A humanized mouse model that supports reticulocyte production and maintenance could provide an alternative method to study *P. vivax* biology and pathogenesis. Furthermore, for decades it has been assumed that *P. vivax* relapse is caused by dormant liver stages called hypnozoites, although no formal proof exists for hypnozoites as the source of relapsing blood stage infection (Galinski *et al.*, 2013), and *in vivo*, *P. vivax* hypnozoites have only been visualized in chimpanzees (Krotoski *et al.*, 1982). Humanized mouse models which support humanized liver tissue could help unravel hypnozoite biology and the phenomenon of relapse in *P. vivax* infections.

Conclusions

Studying the complex life cycle and unique aspects of the biology of human malaria parasites requires the development of specialized tools. In recent years development of humanized mouse models have allowed us to broaden our biological knowledge of specific stages of the *P. falciparum* life cycle. They also start to enable an effective platform for the assessment of pharmacologic and immunological interventional strategies during both pre-erythrocytic and blood stage infection. Further development of these models could provide a combined model of distinct phases of the malaria life cycle from initial delivery by mosquito bite to transmission of sexual stages to the next mosquito. Moving forward, these animal models could well constitute a critical component in the toolbox that will allow for an improved understanding of *Plasmodium* biology relevant to human infection, leading to more effective approaches to combat malaria.

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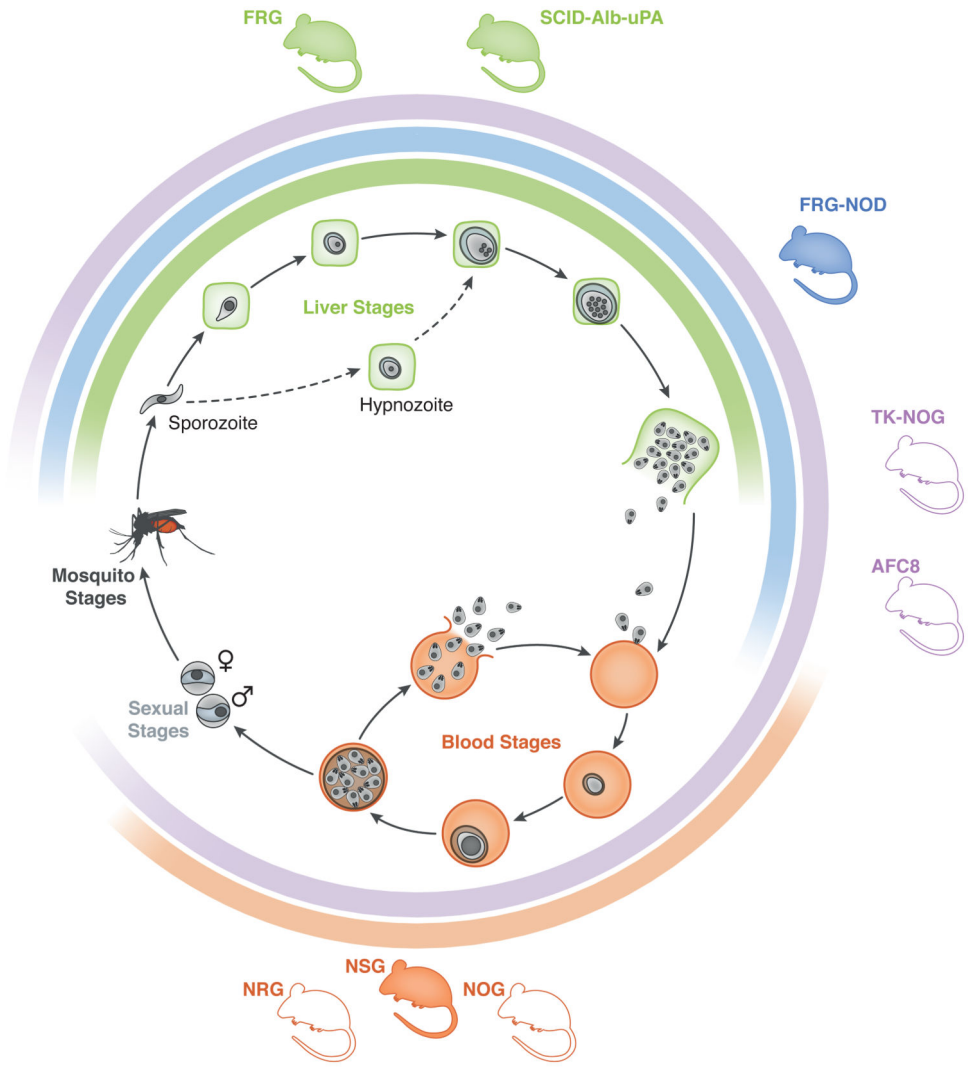

Fig 1. Schematic of humanized mice and their utility to model human malaria parasite life cycle stages

The different stages of the life cycle of the parasites in the human host are illustrated in the center. The lines surrounding it indicate the extent of the cycle covered by each humanized mouse model, color coded to represent the parts of the cycle covered as follows: from mosquito delivery of sporozoites into the skin to release of exo-erythrocytic merozoites from liver (green, FRG and SCID-Alb-uPA); from mosquito delivery of sporozoites into the skin to erythrocyte invasion (blue, FRG-NOD); from mosquito delivery of sporozoites into the skin to sexual stages (purple, TKG-NOG and ACF8); and from erythrocyte invasion to sexual stages (orange, NSG, NRG and NOG). Filled mice indicate models with available data for that part of the cycle, whereas outlined mice indicate models with proposed roles in the corresponding part of the cycle. Mice shown in Green, Blue and purple have been demonstrated to support repopulation with human hepatocytes. Models depicted in purple and orange have been shown to support long-term maintenance of human erythrocytes. The FRG-NOD mouse can support short term repopulation with human erythrocytes, although to our knowledge long term studies have not been attempted.

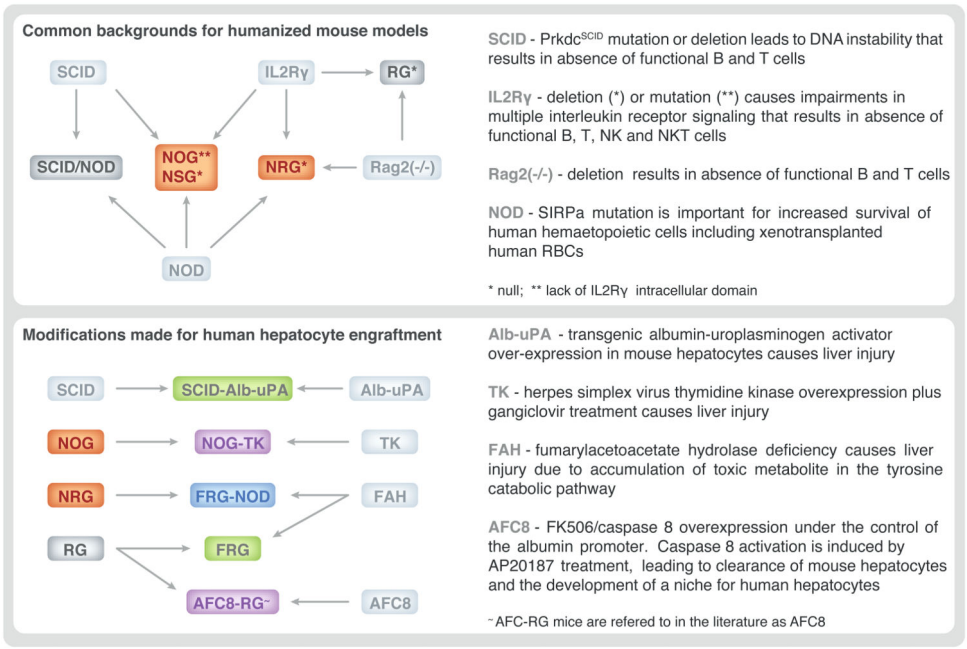

Fig 2. Genealogy of available humanized mice

Backgrounds common to strains of mice that humanized mice models described in this review are depicted, including crosses that resulted in the different strains. Mice shown in Green, Blue and purple have been demonstrated to support repopulation with human hepatocytes. Models depicted in purple and orange have been shown to support long-term maintenance of human erythrocytes. The top panel describes mice with immune compromised status which supports human tissue engraftment. The bottom panel describes the use of transgenes which provide suitable liver damage to establish niche that facilitates human hepatocyte engraftment.

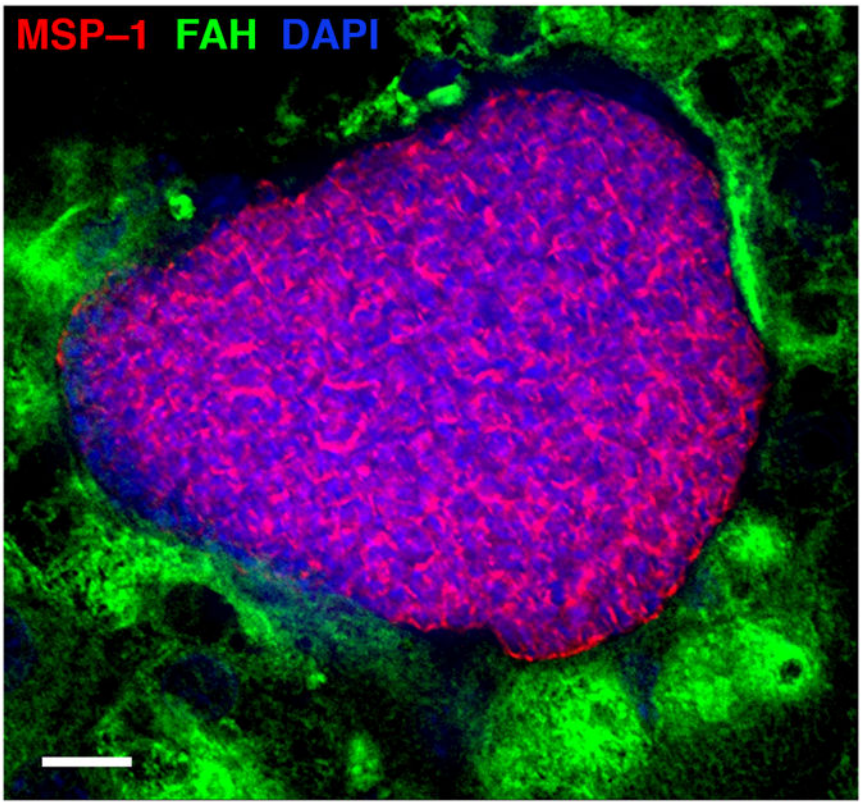

Fig 3. FRG huHep mice support development of *P. falciparum* liver stages

Liver sections of FRG huHep mice at day 7 after *P. falciparum* sporozoite infection were stained with antibodies specific to *P. falciparum* merozoite surface protein 1 (MSP-1; in red) and to human Fumarylacetoacetate Hydrolase (FAH; in green) and DAPI (blue) to visualize DNA. A large liver stage schizont is shown before maturation and differentiation into exo-erythrocytic merozoites. Scale bar – 10 μm.