Plasmodium falciparum infection in "humanised liver" mice

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1 Introduction

Up to the present, the primary model extensively used for malaria research in animals has been the mouse model, mainly because of the availability of rodent malaria parasites. However, there are substantive differences between the biology of rodent malaria parasites and human malaria parasites, including the most deadly, *Plasmodium falciparum*. Furthermore, current *in vitro* systems do not adequately provide us with definitive answers needed for optimal clinical development of new drugs and vaccines against malaria. In this regard, an animal model that can be used with human malaria parasites is urgently needed. However, *P. falciparum* sporozoites do not invade and develop in murine hepatocytes. Therefore, in order to make mice susceptible to *P. falciparum* infection and be able to accommodate development of this parasite, such mice must possess human liver cells. In this study we sought to generate "humanised liver" mice capable of supporting development of *P. falciparum* sporozoites by engrafting human hepatocytes into uPA-NOG mice [expressing transgenic urokinase-type plasminogen activator (uPA) in NOD/Shi-scid IL2Rg(null) mice (NOG)] [1], which have defective murine hepatocytes, in addition to being highly immuno-deficient.

2 The idea

The establishment of "humanised liver" mice capable of supporting the full development of the liver stages of human malaria parasites will provide us an invaluable opportunity to study various aspects of human malaria without the use of human volunteers. This would include investigating the biology of human malaria parasites *in vivo* and testing the potency of various agents against human malaria infection. Furthermore, "humanised liver" mice would give us the means by which efficient and economical precursor analysis can be done prior to the more expensive human clinical trials.

3 Results

In this study (as part of round 3 GCE exploration grants, 2009), we sought to establish a mouse model that can support the development of the liver stages of the human malaria parasite *P. falciparum*. Because *P. falciparum* sporozoites (PfSPZ) can only invade and grow within human hepatocytes, it is imperative to generate "humanised liver" mice if we are to have a non-human animal model. These "humanised liver" mice were made by engrafting human hepatocytes into uPA-NOG mice, generated and bred at the Central Institute for Experimental Animals (CIEA) in Japan. In addition to being highly immuno-deficient, these uPA-NOG mice also have defective murine hepatocytes [1]. The severely immunodeficient NOG background enables higher xenogeneic cell engraftment and donor xenogeneic human hepatocytes can be easily transplanted into young uPA-NOG mice [1]. Furthermore, the absence of neonatal lethality enables mating of homozygotes and thus easy availability of uPA-NOG mice.

We determined the effectiveness of the human hepatocyte engraftment by challenging the engrafted uPA-NOG mice with PfSPZ to see if these human malaria parasites would invade the humanised mouse liver and develop into mature hepatic stages, as outlined in the experimental scheme in Fig. 1. We used four uPA-NOG mice that were 4-7 weeks old. Three uPA-NOG mice were infused intrasplenically with primary hepatocytes from the same human donor [1], and one uPA-NOG mouse was not engrafted. Ten weeks after the engraftment, the level of human albumin in the sera was determined as described [1]. Two uPA-NOG mice (No. 3 and No. 4 in Fig. 2), which received human liver engraftment, produced 3 mg/ml of human albumin in their sera, whereas one uPA-NOG

Figure 1. Schematic of the establishment of "humanised liver" mice and the evaluation of the infectivity of *P. falciparum* sporozoites in these livers. uPA-NOG mice were first engrafted with human hepatocytes. Twelve weeks later, engrafted, as well as intact uPA-NOG mice were challenged with PfSPZ. Six days after challenge, the livers were removed and subjected to real-time qRT-PCR assay and immunohistochemical analysis.

mouse (No. 2 in Fig. 2) had 1.5 mg/ml of human albumin in the sera (data not shown), indicating the rate of successful human liver engraftment to be 40% and 20% of the entire mouse liver, respectively (Fig. 2) [1]. One uPA-NOG mouse (No. 1 in Fig. 2) that did not receive the human liver engraftment and did not have any detectable human albumin in its serum, was used as a negative control. Fourteen weeks after the human liver engraftment, both engrafted mice, as well as the intact uPA-NOG mouse were challenged intravenously with aseptic, purified, cryopreserved, PfSPZ produced at Sanaria Inc. at the dosages of 1.2 x 10^5 to 3.6 x 10^5 PfSPZ per mouse. One uPA-NOG mouse engrafted with human liver and one intact uPA-NOG mouse were injected with 1.2 x 10^5 PfSPZ, and 3.6 x 10^5 PfSPZ were administered to another uPA-NOG mouse engrafted with human liver (Fig. 2). The one uPA-NOG mouse having human liver at 20% of the whole mouse liver was not inoculated with PfSPZ. Six days after injection with PfSPZ, we collected the liver from the infected and non-infected mice, and performed two different assays, as indicated in Fig. 1.

In the first set of experiments, we performed a real-time quantitative RT-PCR using primers specific for *P. falciparum* 18S rRNA to determine the total amount of *P. falciparum* parasites in the liver. This real-time qRT-PCR is adopted from the methodology we previously refined and used for rodent malaria parasites [2]. Briefly, total RNA was extracted from liver samples using Trizol reagent (Invitrogen, Grand Island, NY), and purified RNA was transcribed to cDNA using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. In order to carry out a real-time PCR to determine the level of expression of *P. falciparum*-specific 18S rRNA, we designed a specific set of primers: CTTTTGAGAGGTTTTGTTACTTTGAGTAA (F) and TATTCCATGCTGTAGTATT-CAAACACAA (R) [3]. We also designed a second set of primers: GAAGGTGAAGGTCGGAGTC (F) and GAA-GATGGTGATGGGATTTC (R) in order to detect human GAPDH, as a housekeeping gene. Real-time PCR was then performed using an ABI Prism 7500 Fast Sequence Detector (Applied Biosystems). The reaction was run in duplicates and consisted of 10 µl iTaq FAST SYBR Green supermix with ROX (Applied Biosystems), 1 µl (0.2 µM) of each primer, 2 µl of cDNA, and water up to a total volume of 20 µl. The cycling conditions comprised an initial step at 95ºC for 20 s, followed by 40 cycles at 95ºC for 3 s, and annealing and extension at 60ºC for 30 s. Dissociation curve analysis was implemented to ensure the presence of a single peak at the correct melting temperature. The CT values for 18S rRNA were normalised with GAPDH CT values for each sample, which were expressed as the ratio of 18S rRNA and GAPDH from the duplicates of two independent reactions.

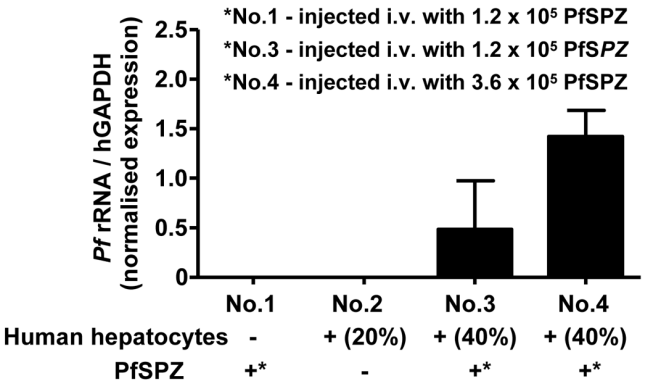

Figure 2. Development of *P. falciparum* liver stages in "humanised liver" mice. Six days after challenge with live PfSPZ, total RNA was extracted from the liver and the amount of parasite-specific rRNA in the liver was measured by a real-time PCR. The numbers of RNA copies were calculated by relative quantification based on endogenous GAPDH RNA. The livers from uninfected, human liver engrafted uPA-NOG mice, as well as from infected uPA-NOG mice without human liver engraftment, were used as a negative control.

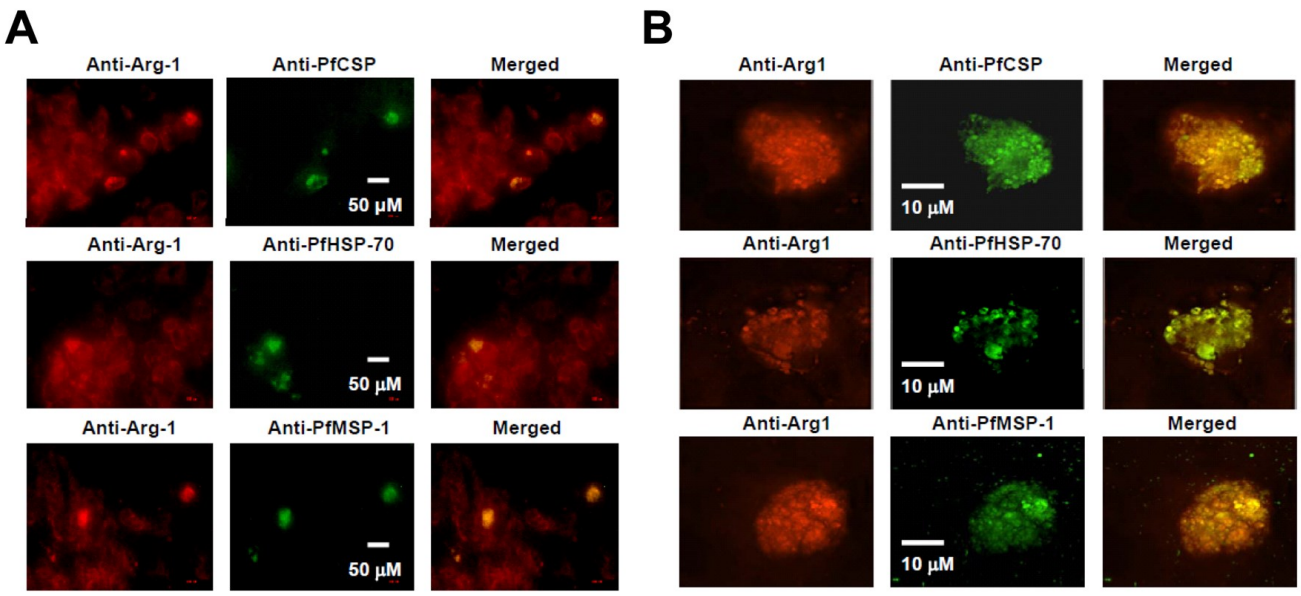

Figure 3. Identification of *P. falciparum* liver stages by immunohistochemical analysis, using monoclonal antibodies against the liver stage. Six days after challenge with PfSPZ, frozen liver sections were prepared from human liver-engrafted uPA-NOG mice that received 3.6 x 10^5 infective PfSPZ. Immunohistochemial analysis was performed using antibodies against human hepatocyte markers and major liver stage antigens of *P. falciparum*. Images were acquired at 800X and 2,000X magnifications.

Through the qRT-PCR, we were able to detect a significant amount of the parasite load in the liver, which was directly correlated with the number of PfSPZ inoculated (Fig. 2). In particular, in the humanised liver mouse (No. 4) challenged with 3.6 x 10^5 live PfSpZ, we detected a higher level of the amount of *P. falciparum*-specific 18S rRNA than in the whole liver of mouse No. 3, which received 1.2 x 10^5 live PfSPZ. It is noteworthy that in the liver of uPA-NOG mouse (No. 1), which did not receive the human liver engraftment, we failed to detect any *P. falciparum*-specific rRNA by the sensitive qRT-PCR.

In the second set of experiments, we performed an immunohistochemical (IHC) analysis to confirm the presence of liver stage malaria parasite [4,5]. For this purpose, we first obtained frozen liver sections from a few small pieces (approx. 1mm^3) of the liver obtained from mouse No. 4 (Fig. 2) on day 6 after PfSPZ challenge (Fig. 1). Briefly, after extensively washing the liver pieces with cold PBS and delicately drying with a paper towel, the tissues were embedded in optimal cutting temperature compound 4583 (Tissue tek; Sakura Finetek USA, Inc. Torrance, CA) and frozen gradually in a bath of ethanol and dry ice. After being frozen, the blocks were cut with a microtome (Microm HM500; Microm International GmbH, Walldorf, Germany) at 10mm of thickness and slides were fixed with cold acetone for 10 min. The slides were hydrated with PBS for another 10 minutes and permeabilised with Perm Buffer (BD Biosciences, San Jose, CA) for 15 min. Following this, they were blocked with 1% BSA in Perm Buffer with 10% normal goat sera for 1 hr and washed three times with Perm/Wash Buffer (BD Biosciences). The slides were then incubated with purified rabbit antibodies against human arginase -1 (Arg-1)(Biolegend, San Diego, CA)(6), or purified monoclonal antibodies against *P. falciparum* CS protein (PfCSP)(7), *P. falciparum* major surface protein-1 (PfMSP-1)(8), or *P. falciparum* heat-shock protein 70 (PfHSP-70)(9) for 2 hr at 4 ºC. After incubation, slides were washed three times and incubated with secondary antibodies, which included Alexa fluor 488 goat anti-mouse IgG (Invitrogen) or Alexa fluor 555 goat anti-rabbit IgG (Invitrogen), for 1 hr at room temperature in the dark. Finally, slides were washed three times and mounted with fluorescent mounting media (KPL, Gaithersburg, MD). These slides were examined with an Olympus IX-70 inverted microscope (Olympus America, Hauppauge, NY) equipped with a Deltavision camera (Applied Precision, Issaquah, WA). Images were taken at 800X and 2,000X magnifications, and processed with Imaris software (Bitplane Inc., South Windsor, CT).

Figure 3A shows that a large proportion of the liver of the "humanised liver" mice consists of human hepatocytes, as stained in red by anti-Arg 1 antibody; and among them, several exo-erythrocytic forms (EEFs) of *P. falciparum* are identified through positive staining using antibodies against major liver stage antigens that include PfCSP (early liver stage), PfHSP-70 (mid to late liver stage) and

PfMSP-1 (late liver stage). The higher magnification photos show the intra-hepatic distribution of the PfCSP, PfHSP-70 and PfMSP-1 by the respective antibody (Fig. 3B). The staining with antibodies against PfCSP and PfMSP-1 show a diffused pattern of distribution, which is well merged with the staining by anti-Arg 1 antibody, whereas the distribution of PfHSP-70 is patchy and distinct.

4 Discussion

In summary, our current study has shown that PfSPZ introduced into the blood stream of our "humanised liver" mice successfully reached and invaded the engrafted human hepatocytes and fully developed into late liver stage parasites in these mice. Unlike the few previous reports demonstrating the presence of low numbers of the EEFs in mice having only a modest degree of engrafted human liver [4,5], our "humanised liver" mice had replaced approximately 40% of the murine liver with human hepatocytes. Consequently, our "humanised liver" mice were found to be adequate in supporting the full development of large numbers of the EEFs of *P. falciparum*.

Given the critical need for an animal model suitable for developing and testing new drugs and vaccines against this single most prevalent infectious disease of mankind, we believe that these "humanised liver" mice, which are fully capable of supporting the liver stage development of *P. falciparum* malaria parasites, will prove to be invaluable.

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