International Journal for Parasitology 47 (2017) 913–922

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Characterising the effect of antimalarial drugs on the maturation and clearance of murine blood-stage *Plasmodium* parasites in vivo

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ARTICLE INFO

Article history:
Received 17 January 2017
Received in revised form 18 April 2017
Accepted 23 May 2017
Available online 31 August 2017

Keywords:
Malaria
Plasmodium berghei
Artemisinin
Mefloquine
Clearance
In vivo
Drug action

ABSTRACT

The artemisinins are the first-line therapy for severe and uncomplicated malaria, since they cause rapid declines in parasitemia after treatment. Despite this, in vivo mechanisms underlying this rapid decline remain poorly characterised. The overall decline in parasitemia is the net effect of drug inhibition of parasites and host clearance, which competes against any ongoing parasite proliferation. Separating these mechanisms in vivo was not possible through measurements of total parasitemia alone. Therefore, we employed an adoptive transfer approach in which C57BL/6J mice were transfused with *Plasmodium berghei* ANKA strain-infected, fluorescent red blood cells, and subsequently drug-treated. This approach allowed us to distinguish between the initial drug-treated generation of parasites (Gen_0), and their progeny (Gen_1). Artesunate efficiently impaired maturation of Gen_0 parasites, such that a sufficiently high dose completely arrested maturation after 6 h of in vivo exposure. In addition, artesunate-affected parasites were cleared from circulation with a half-life of 6.7 h. In vivo cell depletion studies using clodronate liposomes revealed an important role for host phagocytes in the removal of artesunate-affected parasites, particularly ring and trophozoite stages. Finally, we found that a second antimalarial drug, mefloquine, was less effective than artesunate at suppressing parasite maturation and driving host-mediated parasite clearance. Thus, we propose that in vivo artesunate treatment causes rapid decline in parasitemia by arresting parasite maturation and encouraging phagocyte-mediated clearance of parasitised RBCs.

1. Introduction

There were an estimated 214 million cases of malaria in 2015, resulting in 438,000 deaths (World Health Organization (WHO), 2015b). Artesunate (an artemisinin derivative) is the recommended treatment for patients with complicated malaria, and is associated with improved survival among those admitted to hospital with severe malaria (Dondorp et al., 2010; Newton et al., 2013; World Health Organization, 2015a). However, growing parasite resistance to artesunate and its partner drugs has prompted the search for alternative therapies (Dondorp et al., 2009; Phyo et al., 2012; Amaratunga et al., 2016). When assessing antimalarial efficacy in vivo, key metrics include the time to clearance of parasitemia (Jiang et al., 1982), the parasite reduction ratio (White, 1997; Marquart et al., 2015), and more recently the rate of clearance of parasitemia (Flegg et al., 2011; Abdulla et al., 2015). The usefulness of these metrics became most apparent when they affirmed the use of highly effective artemisinins, which were originally found to elicit very rapid elimination of parasitemia in patients compared with other antimalarial drugs (Jiang et al., 1982; White, 1994; Hien et al., 1996). More recently, the detection of slower declines in parasitemia after treatment with artemisinins played a critical role in the early detection of artemisinin resistance (Dondorp et al., 2009; Phyo et al., 2012).

Despite the well-established utility of these measures for assessing drug efficacy, the underlying mechanisms driving a decline in parasitemia after treatment are not well understood. Here, we hypothesise that a reduction in parasite numbers after

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http://dx.doi.org/10.1016/j.ijpara.2017.05.009
0020-7519/

treatment involves at least two major concurrent processes (Dogovski et al., 2015; Hastings et al., 2015): firstly, an impairment of parasite development that hinders further proliferation (Wilson et al., 2013), and secondly, host-mediated clearance of drug-affected parasites from circulation, most likely by phagocytic cells in the spleen (Chotivanich et al., 2002). Thus far, it has not been possible to separate and measure these two processes, either clinically or otherwise, in vivo.

In this paper, we used a mouse model of infection and an adoptive transfer approach to make quantitative assessments of parasite maturation and host clearance during the first 24 h after treatment with artesunate. This approach allowed us to study a single generation of infected parasites and separate the process of host clearance from parasite maturation and proliferation. We found that the normal progression of parasite maturation was rapidly arrested after treatment, suppressing further parasite proliferation over the first 24 h. We also measured the clearance rate of drug-affected early-stage and late-stage parasites from circulation, and showed that clearance of drug-affected early-stage parasites is largely mediated by host phagocytic cells. Finally, we also explored the same processes after treatment with an alternative antimalarial drug, mefloquine (MQ). Thus, our combined experimental and analytical methodology in mouse models of malaria has permitted greater insight into the in vivo effects of antimalarial drugs on parasite replication and subsequent host clearance.

2. Materials and methods

2.1. Mice and ethics

Female C57BL/6J mice aged 6–12 weeks were purchased from the Animal Resource Centre (Canning Vale, Perth, WA, Australia) and maintained under conventional conditions. This study was carried out in strict accordance with guidelines from The National Health and Medical Research Council of Australia (NH&MRC), as detailed in the document Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition, 2004. All animal procedures and protocols were approved (A02-633M) and monitored by the QIMR Berghofer Medical Research Institute Animal Ethics Committee, Australia.

2.2. Parasites and infections

Plasmodium berghei ANKA (*Pb*A) and *Plasmodium yoelii* XNL *(Py*XNL*)* strains were used in all experiments after a single in vivo passage in wild type C57BL/6J mice (Animal Resource Centre). Transgenic *Pb*A-GFP strains were maintained as previously reported (Haque et al., 2011b). All donor mice were infected with either 10^5 (*Pb*A) or 10^4 (*Py*XNL) infected red blood cells (RBCs) i.v. via the lateral tail vein.

2.3. Adoptive transfer of donor RBCs

The adoptive transfer of donor parasites was carried out as previously described (Khoury et al., 2015). Donor parasitised RBCs (pRBCs) were collected from mice infected with *Pb*A or *Py*XNL by cardiac punctures. Heparinised blood was washed twice in Ca^{2+}/Mg^{2+}-free PBS (PBS-A), and stained in CellTrace™ Far Red DDAO-SE (Life Technologies, USA) according to manufacturer's instructions. Briefly, 50 μg of CellTrace™ were dissolved for 10 min in 25 μl of DMSO. This was added to 5 ml of resuspended blood in PBS-A. Blood was stained in the dark, at room temperature with constant rolling for 15 min, and then washed twice in 10× volumes of PBS-A. Successful labelling of RBCs was confirmed by flow cytometry using an LSRII Fortessa analyzer (BD Biosciences, Australia) and FlowJo software (Treestar, CA, USA). CellTrace™-labelled blood was resuspended in 2 ml volumes per donor mouse, and injected in 200 μl volumes via i.v. injection using a 26 G needle.

2.4. Drug preparation

Sodium artesunate (Guilin Pharmaceutical Co., Ltd., Guilin, Guangxi, China) was prepared according to the manufacturer's instructions, diluted in 0.9% saline (Baxter, Australia), and administered to mice immediately after donor parasites were transfused, and for some mice, a second time 12 h later. Doses ranged from 10 μg to 1000 μg (corresponding to 0.5 mg/kg to 50 mg/kg) and were administered in 200 μl volumes via i.p. injection.

Mefloquine hydrochloride (Lariam®) (Roche, Basel, Switzerland) was dissolved to a stock concentration of 25 mg/ml in ultrapure water (Milli-Q®, Millipore) containing 10% v/v DMSO (Sigma, USA) for 30 min at room temperature with constant agitation. This was further diluted just before administration to mice at doses indicated above, and given via i.p. injection in 200 μl volumes.

2.5. Phagocyte depletion in vivo

Host phagocytes were depleted in vivo with a single i.v. injection (via a lateral tail vein using 26 G needles) of 200 μl of clodronate-containing liposomes (www.clodronateliposomes.com) 3 days prior to transfusion of labelled blood and antimalarial drug treatment.

2.6. In vitro culturing of peripheral blood

Peripheral blood was collected and diluted 1:40 in culture medium (Roswell Park Memorial Institute medium (RPMI), 20% FBS, 1 U/ml of heparin sodium). A 200 μl volume of diluted blood was plated per well in 96-well flat bottom plates (Corning, USA). Plates were covered, then flushed for 30 s with 5% CO_2, 5% O_2, 90% N_2 in a closed secondary container which was then sealed. Cultures were incubated at 37 °C for the specified times. Cells were then resuspended by gentle pipetting immediately before staining for flow cytometric analysis. Separate cultures were maintained for each ex vivo time point.

2.7. Flow cytometric analysis of blood

Forward scatter (FSC) and side scatter (SSC) were used to distinguish RBCs from other cell types. Plotting the FSC-Area (FSC-A) and the FSC–Height (FSC–H) allowed the exclusion of doublets (events recorded by the flow cytometer that are the result of two cells being detected simultaneously). For imaging flow cytometry, the aspect ratio and area of channel 1 (bright field; BF) were used to distinguish RBCs from other cell types and exclude doublets. Focused events were then selected by plotting of the gradient RMS feature of channel 1 (first camera; BF) and the gradient RMS feature of channel 9 (second camera; BF). A flow cytometric method, adapted from various research groups (Apte et al., 2011; Klonis et al., 2011; Malleret et al., 2011), was employed to simultaneously detect adoptively transferred (CellTrace™-labelled) RBCs, to distinguish DDAO-SE^{pos} (donor) from DDAO-SE^{neg} (recipient) RBCs, and to determine the developmental stage of pRBCs (Supplementary Figs. S1, S2). Briefly, a single drop of blood from a tail bleed was diluted and mixed in 200 μl of RPMI medium containing 5 U/ml of heparin sulphate. Diluted blood was simultaneously stained for 30 min in the dark at room temperature with an anti-mouse erythroid cell antigen TER119-APC antibody (2 μg/ml; BioLegend, USA), cell-permeant RNA/DNA stain, Syto84 (5 μM; Life Technologies) and with DNA stain, Hoechst 33342 (10 μg/ml;

Sigma). Staining was quenched with 10 volumes of RPMI medium, and samples immediately analysed by flow cytometry using an LSRII Fortessa analyser (BD Biosciences) and FlowJo software (Treestar). For high throughput imaging, cells were acquired at the lowest flow rate under 60x magnification using an Amnis ImageStream®X (EMD Millipore, USA) and data analysed with IDEAS® Software version 6.1 (EMD Millipore). Adoptively transferred donor RBCs were readily distinguished from endogenous RBCs by CellTrace™-labelling. Infected RBCs were detected as being GFP^{pos} (in the case of the *Pb*A transgenic parasites), Hoechst 33342^{pos} and $Syto84^{pos}$.

2.8. Statistical analysis

All errors reported in this paper are S.E.M. ($\pm\sigma/\sqrt{n}$). All comparisons of group means were performed using a one-way ANOVA followed by a post hoc contrast analysis, unless otherwise stated (using the anova1.m and multcompare.m functions in MATLAB R2014b (8.4.0.150421)). Regression analysis was performed using the fitlm.m function in the Statistics and Machine Learning toolbox in MATLAB R2014b (8.4.0.150421). The confidence intervals (CIs) and the significance of regression coefficients were determined from this inbuilt package, which utilises a Wald test for significance of covariates.

3. Results

3.1. Tracking a single generation of parasites after artesunate treatment in vivo

To begin dissecting in vivo mechanisms of action for artesunate, we first determined whether this drug was indeed effective in mice infected with *Pb*A. Artesunate treatment was initiated at day 5 p.i., when mice start to exhibit symptoms of severe disease including liver damage (Haque et al., 2011a), which led to a rapid decline in parasitemia associated with complete protection from fatal neurological symptoms (Fig. 1A). These data confirmed that artesunate is highly effective at reducing circulating pRBC numbers in mice, thus permitting further studies of in vivo mechanisms of action for this antimalarial drug.

In order to dissect in vivo mechanisms of action, we utilised our recently developed method in which fluorescently-labelled donor pRBCs are infused and tracked in vivo (Khoury et al., 2015). This approach differentiates between one generation of parasites and their progeny, i.e. the donor pRBCs that we transfused into the recipient mice, which we term Gen_0 (Fig. 1B, C), from the progeny of these transfused parasites in unlabelled (i.e. recipient) RBCs, which we term Gen_1 (Fig. 1B, C). We transfused mice with labelled, Gen_0 pRBCs and immediately treated with artesunate or control saline (Fig. 2A). In control-treated mice we observed that Gen_0 pRBCs, as expected, disappeared from circulation over 24 h, most likely due to a combination of sequestration, rupture, and clearance by the host (Deharo et al., 1996; Khoury et al., 2015)), with 41.5% ± 0.9 of the initial concentration of donor pRBCs remaining at 11 h, and 6.4% ± 0.7 remaining after 24 h.

We then analysed the removal of Gen_0 pRBCs after treatment with artesunate in order to measure the increase in parasite clearance due to treatment. Contrary to our initial expectations, mice treated with artesunate showed a dose-dependent increase in the number of Gen_0 pRBCs remaining in circulation compared with control-treated mice (Fig. 2B, C). In mice given 100 μg (5 mg/kg) of artesunate at 0 h and 11 h, almost twice as many Gen_0 pRBCs remained in circulation at 11 h (73% ± 3, $P < 0.0001$, respectively); by 24 h, nearly three times as many parasites remained in circulation (17.6% ± 2.6, $P < 0.01$) (Fig. 2Cb). Since both tissue sequestration and schizont rupture likely contribute to the loss of pRBCs from circulation in untreated mice, our data suggested that artesunate slowed the exit of Gen_0 pRBCs from circulation by hindering or halting parasite maturation.

We next repeated the above experiment with a second rodent-infective species, *P. yoelii* 17XNL strain. Once again, artesunate treatment of *P. yoelii*-infected mice led to a dose-dependent increase in the number of Gen_0 pRBCs remaining in circulation (Fig. 2D), indicating that our observations were not limited to *Pb*A infection. We also repeated these experiments with fivefold higher doses of artesunate (Fig. 2D). This led to further increases in the persistence of Gen_0 parasites (Fig. 2D), which strongly suggested that the phenomenon of drug-induced persistence in circulation was not an artefact of sub-optimal drug dosing.

3.2. In vivo artesunate halts parasite maturation

Given the persistence of Gen_0 pRBCs after treatment, we next sought to assess the effect of artesunate on parasite maturation and survival. To do this, we took peripheral blood from mice at 2 h after donor RBC transfusion and artesunate treatment, and cultured these samples in vitro in the absence of drug, with regular flow cytometric assessments of the progression of Gen_0 pRBCs through their life stages (Fig. 3A). While Gen_0 pRBCs from control-treated mice began to transition to the schizont stage by 6 h of culturing, this only began by 15 h after artesunate exposure (200 μg) (Fig. 3B). By 24 h in culture, 57 ± 2% ($n = 2$) of Gen_0 pRBCs had become schizonts in control-treated mice, versus 11 ± 3% ($n = 2$) after treatment with 200 μg of artesunate. The delay in transition to the schizont stage suggested that at least some, but perhaps not all, parasites were impaired in their capacity to mature when exposed to 2 h of 200 μg of artesunate treatment in vivo. To determine the effect of longer exposure times and increased dosing, we performed the same experiment after 6 h in vivo exposure to 200 μg or 1000 μg of artesunate compared with saline controls (Fig. 3C). Six hours of exposure to 200 μg of artesunate in vivo led to greater impairment of parasite development, although a small proportion of Gen_0 pRBCs still reached the schizont stage by 24 h in culture (22 ± 3%, schizonts at 24 h in culture compared with 11 ± 1% initially, Fig. 3C). After exposure to 1000 μg of artesunate, Gen_0 pRBCs showed no discernible progression through their life stages by 24 h in culture. Together, our data suggested that if exposed to a high enough dose of artesunate, all parasites were rapidly and severely impaired in their capacity to mature after 6 h of in vivo exposure in this experimental model.

To confirm in our laboratory the use of established flow cytometric methodology for assessing parasite life cycle stages, we employed a high-throughput imaging cytometry approach (Amnis ImageStream®) in which RBCs passing through a flow cytometer were simultaneously photographed to allow their direct visualisation, thus allowing us to assess the validity of our flow cytometric gating strategy. Imaging cytometry was performed on cultures of parasites that had been recovered from infected mice treated with either saline or artesunate (1000 μg; Supplementary Fig. S1), as well as directly in vivo (without drug treatment) (Supplementary Fig. S2). These experiments confirmed that our flow cytometric gating strategies correctly distinguished between early- and late-stage parasites, since pRBCs staining most strongly for Hoechst 33342 by flow cytometry also showed punctate DNA staining consistent with schizogony having occurred. Imaging cytometry, at the resolution performed in this study, revealed no striking differences between ring-stage parasites from mice treated with either saline or artesunate (Supplementary Fig. S1).

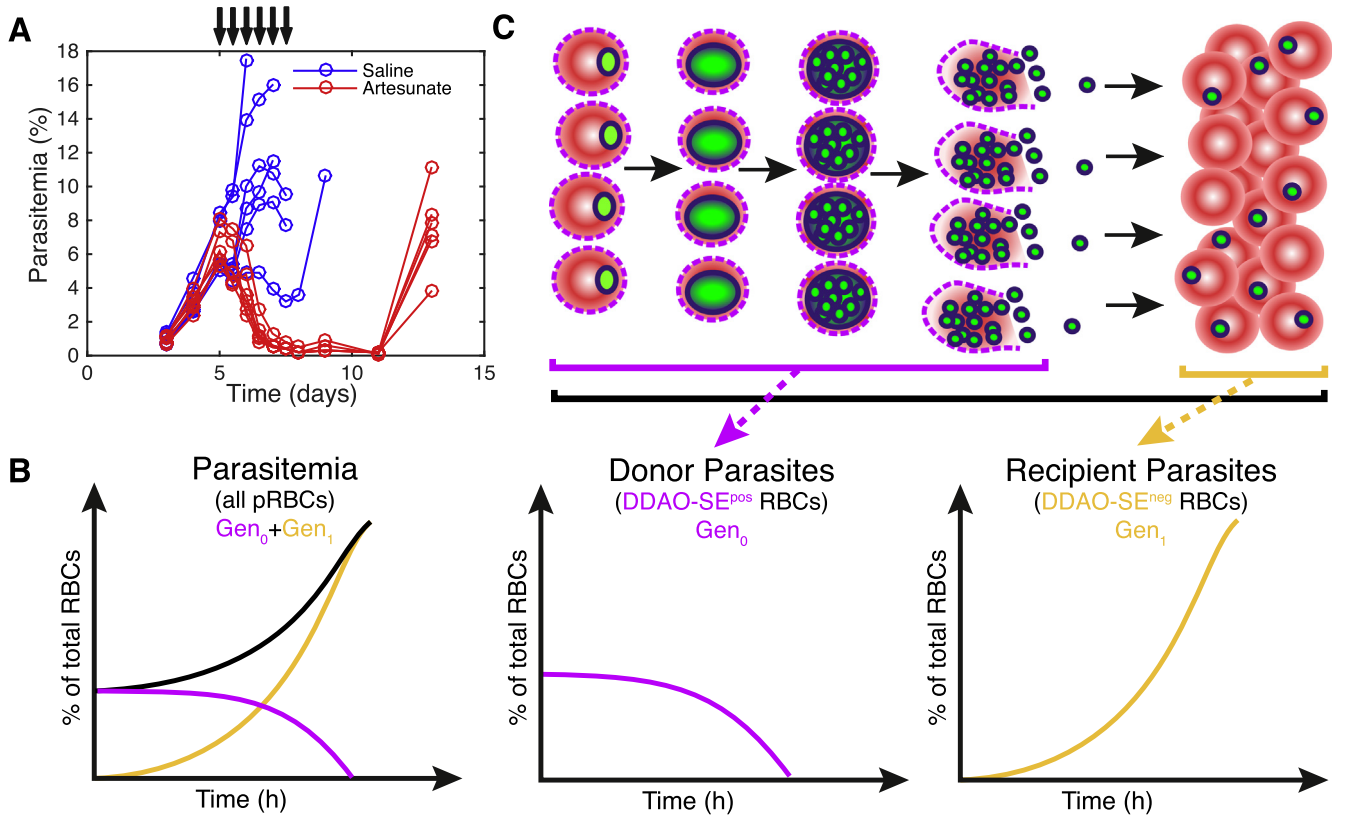

Fig. 1. Deconvoluting the parasitemia curve using fluorescent labelling. (A) Effectiveness of artesunate in vivo. The time course of *Plasmodium berghei* ANKA strain parasitemia in the saline control (blue (black)) and artesunate-treated (red (grey)) mice in vivo. Parasitemia was measured daily using flow cytometry until day 5 of infection, and every 12 h during treatment beginning from day 5 with sodium artesunate (200 μg doses, i.e. 10 mg/kg, every 12 h for 2.5 days (indicated by arrows)). All of the control mice received clinical illness scores that required that they be sacrificed before day 13 (6/6), whereas none of the treated mice showed serious illness by day 13 (0/6). (B) Schematic diagram illustrating how parasitemia is composed of multiple generations of parasites. (C) Schematic diagram illustrating how fluorescent labelling can be used to distinguish between these different generations of parasites (Gen_0, donor parasitized red blood cells; Gen_1, recipient pRBCs). RBC, red blood cells; DDAO-$SE^{pos/neg}$, indicates those cells that were and were not labelled with CellTrace™ Far Red DDAO-SE (Life Technologies, USA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Estimating the stage-specific clearance rate of treated parasites

Since all measurable parasite maturation was disrupted after 6 h of treatment with 1000 μg of artesunate, we next considered the fate of drug-impaired parasites in vivo. We first noted that very few Gen_1 pRBCs were generated in mice throughout the first 24 h after treatment with 1000 μg of artesunate, consistent with the complete suppression of Gen_0 pRBCs in vitro (Fig. 3C). This confirmed that a single high dose of artesunate in vivo blocked almost all measurable parasite maturation, rupture and RBC re-invasion by 6 h and up to 24 h after treatment. Importantly, therefore, we reasoned that any loss of pRBCs observed during this window was the direct result of host-mediated clearance of drug-impaired parasites or lysis of drug-affected pRBCs (both of which we refer to as clearance). To measure clearance of these drug-impaired parasites, we fitted the rate of decline of Gen_0 pRBCs from 6 h – 24 h in mice treated with 1000 μg of artesunate, and found that drug-impaired parasites were removed with a half-life of 6.7 h (95% Confidence Interval (CI): 6.2 to 7.3 h) (Fig. 4B). Furthermore, we distinguished early-stage (ring and trophozoite) Gen_0 pRBCs from late-stage (schizont) Gen_0 pRBCs (Fig. 3B), and estimated the clearance rate of each life stage independently. Drug-affected schizonts (half-life: 4.7 (95% CI: 4.2 to 5.3) h) were cleared significantly faster than drug-affected early stages (half-life: 7.0 (95% CI: 6.4 to 7.7) h) ($P < 0.0001$, Wald test).

Next, we examined the role of host phagocytes in mediating clearance of drug-affected parasites, by depleting these cells with clodronate-containing liposomes. This method for depleting phagocytes is well established (Baer et al., 2007; Couper et al., 2007; Arnold et al., 2010; Ishida et al., 2013; Tavares et al., 2013; Borges da Silva et al., 2015; Imai et al., 2015; Mimche et al., 2015; Fontana et al., 2016; Terkawi et al., 2016). In fact a recent study used clodronate liposomes to reveal a key role for splenic dendritic cells in clearing parasites in mice (Borges da Silva et al., 2015). Hence, we first confirmed successful depletion of phagocytes by flow cytometric assessment of splenic dendritic cells, using cell surface MHC-II and CD11c as established markers (Supplementary Fig. S3). Following clodronate liposome treatment we adoptively transferred and tracked labelled pRBCs. The same fitting of Gen_0 pRBC populations in these mice showed that clodronate treatment led to a 59% (95% CI: 48% to 70%) reduction in the clearance rate of drug-impaired parasites ($P < 0.0001$). In particular, we noted that early-stage parasites were cleared much more slowly in clodronate-treated mice (2.7-fold increase in half-life compared with phagocyte-replete mice, $P < 0.0001$), while schizonts were cleared only marginally more slowly in clodronate-treated mice (1.4-fold increase in half-life compared with intact mice, $P < 0.0001$). Together, these results indicate that host phagocytes play an important role in clearing artesunate-affected pRBCs, particularly of younger developmental stages.

3.4. Effects of MQ on P. berghei in vivo

After characterising the impact of artesunate on parasite replication and clearance, we next explored whether these observations were evident with other drugs. We repeated our adoptive transfer

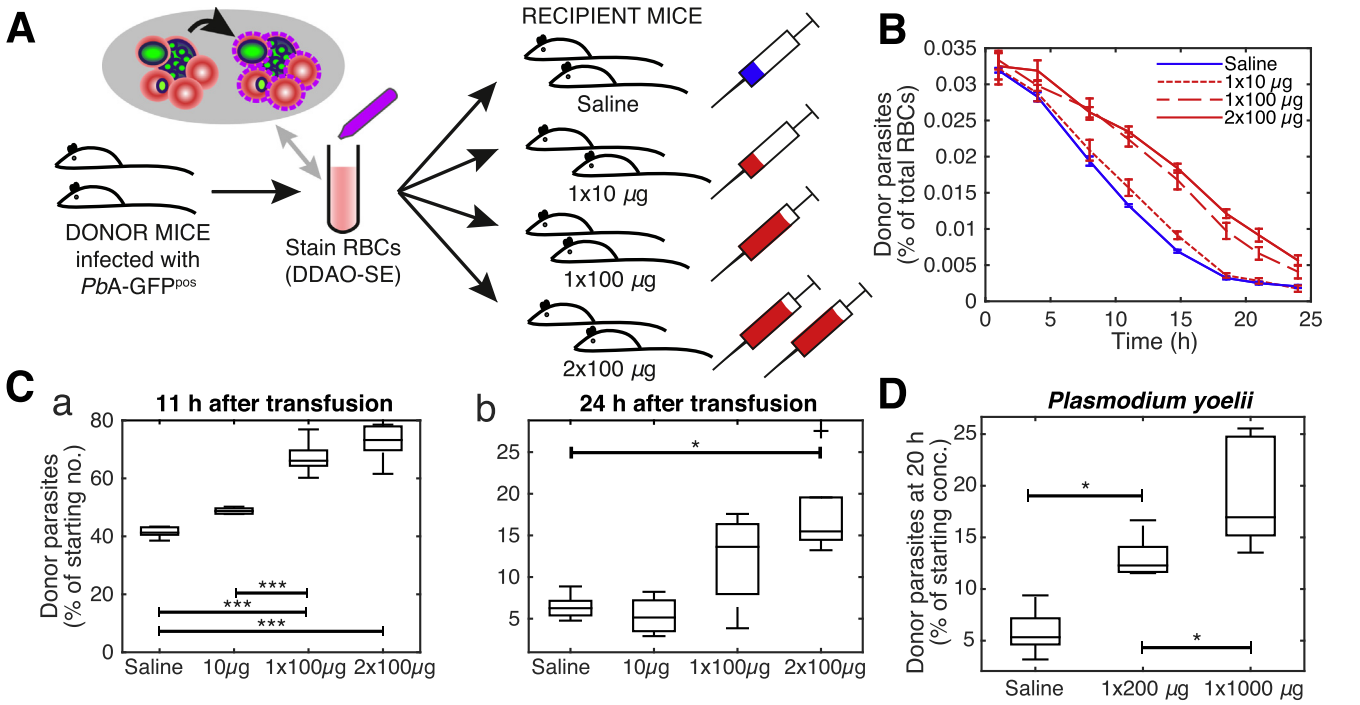

Fig. 2. Artesunate causes parasitized red blood cells to persist in circulation. (A) The adoptive transfer protocol. Two donor mice (C57BL/6) infected with *Plasmodium berghei* ANKA strain (*Pb*A) were bled to provide donor pRBCs. The donor blood was pooled together and stained with CellTrace™ Far Red DDAO-SE (Life Technologies, USA) before being transfused into four groups of mice ($n = 5$ each) that received either saline (i.p.), a single dose of either 10 μg or 100 μg of artesunate, or two doses of 100 μg of artesunate (corresponding to 0.5 mg/kg, 5 mg/kg or 2 × 5 mg/kg). Blood samples were collected from these mice at 1, 4, 8, 11, 15, 18.5, 21, 24 and 48 h after transfusion for flow-cytometry analysis. (B) The concentration of transfused parasites (labelled RBCs that contain parasites) as a percentage of total RBCs. Untreated mice (solid blue (black)), and mice treated with 10 μg (red (grey), short dashes), 100 μg (red (grey), long dashes) and 2 × 100 μg (solid red (grey)) of artesunate are shown. (Ca, Cb) The median percentage of transfused parasites remaining at 11 and 24 h after transfusion, respectively, compared with 1 h after transfusion. The centre line indicates the median, the box indicates the 25th and 75th centiles and the bars show the range. We observed a dose-dependent increase in donor *Pb*A pRBCs remaining in circulation after treatment with artesunate. (D) Dose-dependent increase in donor non-lethal *Plasmodium yoelii* XNL pRBCs remaining in circulation after treatment. Three groups of C57BL/6J mice ($n = 5$ each) received pRBCs infected with *P. yoelii*. Mice were immediately treated with saline (no drug group), a single 200 μg dose of artesunate i.p., or a single 1000 μg dose of artesunate. The percentage of donor parasites remaining at 20 h after transfusion was higher in the 200 μg group compared with the control ($P < 0.05$, using a post hoc contrast analysis), and higher in the 1000 μg group compared with the 200 μg group ($P < 0.05$, using a post hoc contrast analysis). ($^{*}P < 0.05$ and $^{***}P < 0.0005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiments and treated mice with various doses of MQ. A single dose of MQ also caused a decline in total parasitemia (Fig. 5B). Similar to artesunate, we saw a dose-dependent increase in the number of Gen_0 pRBCs remaining in circulation at 24 h after MQ treatment compared with controls (Fig. 5A). However, unlike artesunate, even high doses of MQ were unable to completely arrest progression to Gen_1 over the first 24 h (Fig. 5B).

Next we performed an adoptive transfer experiment with higher doses of MQ, and studied parasite maturation in vitro after 7 h of in vivo drug treatment. In blood from control-treated animals 16 ± 1% of Gen_0 pRBCs were schizonts at the start of culturing, which increased to 67 ± 1% after 24 h of culturing. Treatment with 500 mg/kg of MQ significantly reduced the proportion of Gen_0 pRBCs progressing to the schizont stage in vitro (22.7 ± 0.5% after 24 h culture) ($P < 0.0001$). However, even with this high dose of MQ, ~9% of Gen_0 pRBCs continued to mature into schizonts by 24 h (from 13.9 ± 0.3% to 22.7 ± 0.5%) (Fig. 6A). Taken together, our data suggested that MQ was less efficient than artesunate at halting progression of Gen_0 pRBCs to the next generation in vivo.

3.5. MQ-impaired parasites are not removed by the host as rapidly as artesunate-impaired parasites

Finally, we directly compared in vivo clearance of MQ- or artesunate-treated pRBCs, by analysing the rate of removal of Gen_0 pRBC parasites from 6–24 h in mice treated with either 50 mg/kg of artesunate (excluding one mouse in which treatment was ineffective) or 500 mg/kg of MQ (Fig. 6C). In our assay, Gen_0 pRBCs are likely to disappear from circulation after treatment for two reasons; (i) they mature and rupture, and (ii) because they are affected by drug and then removed by the host or potentially lyse spontaneously. As noted above, artesunate inhibits all measurable parasite development so we could exclude the first possibility. However, unlike artesunate, even at high doses MQ did not inhibit all measurable parasite development. Therefore, even though after MQ treatment Gen_0 pRBCs are disappearing due to both options listed above (i.e. (i) rupture and (ii) clearance) they are still disappearing more slowly than after treatment with artesunate (where only option (ii) – clearance – is at play) (half-life 13.0 h versus 9.1 h respectively, $P < 0.001$, Wald test). This confirms that MQ-treated parasites must be cleared significantly more slowly than artesunate-treated parasites. It follows that not only did artesunate impair a greater proportion of Gen_0 pRBCs within the first 7 h of treatment, but artesunate-affected pRBCs were also removed from circulation much faster than MQ-affected pRBCs.

4. Discussion

The overall decline in parasitemia after treatment with an antimalarial drug has been used as a critical measure of drug efficacy in vivo (Jiang et al., 1982; Dondorp et al., 2009; Flegg et al., 2011). In this study we dissected and measured the underlying mechanisms of drug-mediated control of parasitemia in vivo. We established that the overall decline in parasitemia is the net effect

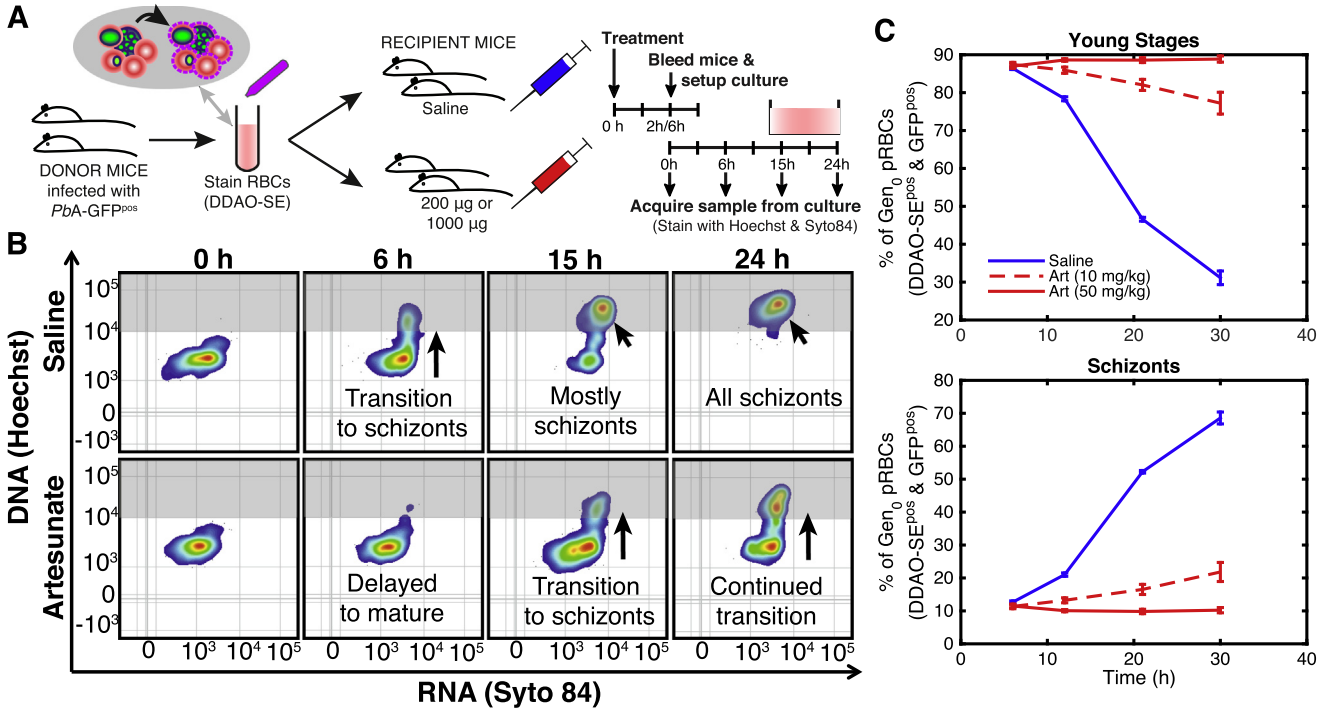

Fig. 3. Artesunate impairs parasite maturation. (A) In order to examine the effect of artesunate exposure to *Plasmodium berghei* ANKA (*Pb*A) strain parasite maturation more directly, blood was taken from mice 2 or 6 h after mice were transfused with donor blood and treated with artesunate. This blood was cultured and sampled regularly. (B) Using DNA and RNA staining and flow cytometry, the developmental stage of drug-exposed parasitized red blood cells could be assessed (Supplementary Figs. S1, S2). A significant proportion of pRBCs from mice that were treated with 200 µg of artesunate had not yet become schizonts by 24 h. Artesunate treatment impaired pRBC development, but some pRBCs were still able to mature after 2 h of in vivo exposure to 200 µg of artesunate. (C) Quantifying the proportion of early-stage and late-stage parasites in culture in a second experiment in which a third group of mice received a higher dose of artesunate (1000 µg or 50 mg/kg), and in which blood was taken from mice 6 h after treatment, revealed that a sufficiently large dose of artesunate was capable of preventing all measurable progression of pRBCs to the schizont stage.

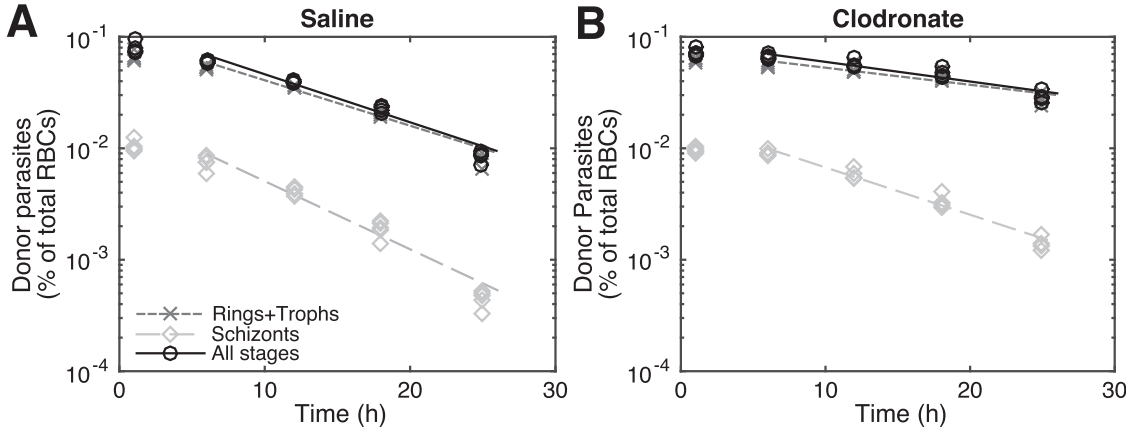

Fig. 4. Clearance rate of artesunate-effected parasitized red blood cells (pRBCs) by life stage, with and without phagocyte depletion. (A) Fitting the decline of donor pRBCs (Gen_0) pRBCs from 6 to 24 h provides an estimate of the rate of removal of artesunate-effected pRBCs. DNA and RNA staining allows life-stage assessment, and hence clearance rate assessment of the early and late-stage parasites. (B) The same analysis was performed in mice depleted of phagocytic cells using clodronate. Trophs, trophozoites.

of at least three processes: the efficacy of drug-action to inhibit parasite development (Fig. 3), any ongoing replication of parasites (Fig.5B), and the rate of removal of drug-affected parasites by the host (Fig. 4). Our assays also provide a possible means for preclinical dissection of these mechanisms for any candidate antimalarial treatment.

We observed that high doses of artesunate were able to fully suppress parasite development in the first 24 h after treatment, even when cells were removed from mice at 6 h and cultured in vitro in the absence of drug. Moreover, these drug-affected parasites were subsequently cleared from circulation with a half-life of 6.7 h. However, it did not appear that all parasites were 'killed' by a single high dose of artesunate, since parasite replication was again evident in treated mice beyond the 24 h period of observation (Fig. 5B). We were not able to characterise the status and proportion of parasites that contributed to further replication (Supplementary Fig. S1). It is possible that a proportion of parasites was completely unaffected by treatment, or that a proportion of parasites survived but was in some way impaired by therapy. Later replication appeared to occur at a normal rate, consistent with no lasting effects of artesunate on at least some parasites. These data appear consistent with other observations in vitro and in vivo,

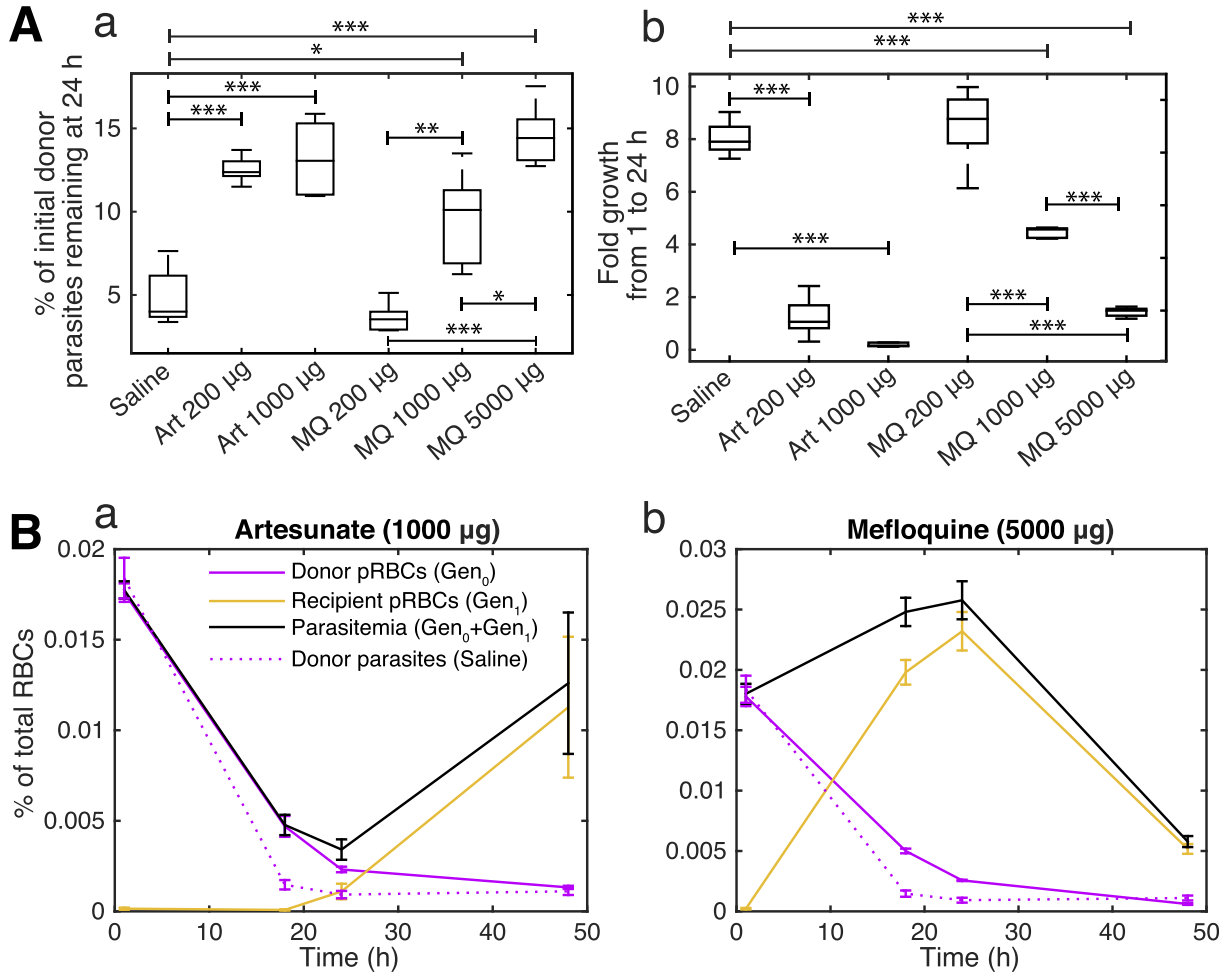

Fig. 5. Mefloquine causes parasites to persist in circulation. (Aa) The donor parasites remaining at 24 h after transfusion as a percentage of the concentration of donor parasites 1 h after transfusion in mice given saline, 200 µg (10 mg/kg) of artesunate (Art), 1000 µg (50 mg/kg) of artesunate, 200 µg (10 mg/kg) of MQ, 1000 µg (50 mg/kg) of mefloquine or 5000 µg (250 mg/kg) of MQ. (*P* values calculated using post hoc contrast analysis, $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$). (Ab) The fold growth in parasitemia over the same time window. (B) The concentration of donor parasitized red blood cells (Gen_0), recipient pRBCs (Gen_1) and the total parasitemia ($Gen_0 + Gen_1$) in mice receiving (Ba) 1000 µg of artesunate and (Bb) 5000 µg of mefloquine. (Ba) We observed that the decline in parasitemia following artesunate treatment was equivalent to the decline in the labelled donor parasites in the first 18 h after treatment, parasitemia begins to increase after 18 h. (Bb) We observed that the total parasitemia increased immediately after treatment, but declined between 24 and 48 h post treatment. The decline in donor parasites in saline control groups is shown for reference (dotted lines), illustrating that both drugs caused donor parasites to persist in host circulation.

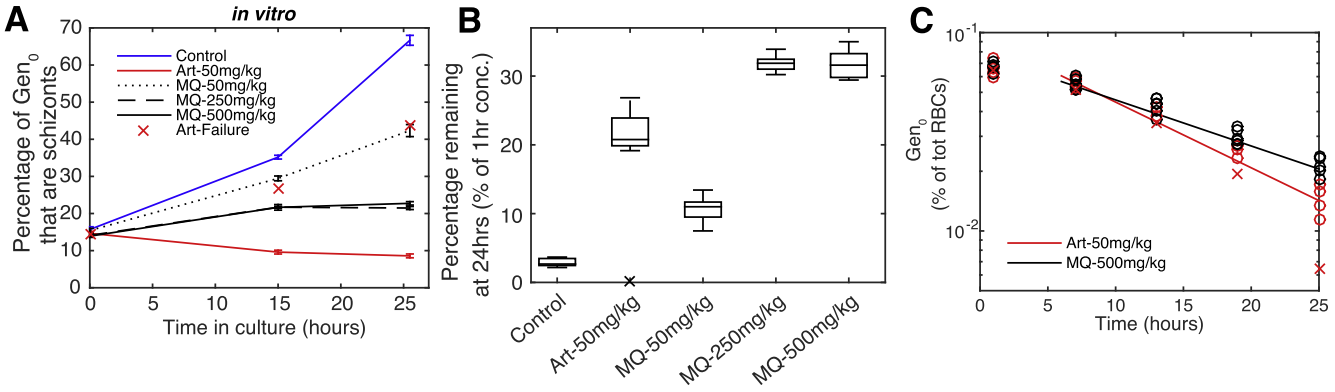

Fig. 6. Mefloquine-affected parasitized red blood cells are not cleared as quickly as artesunate (Art)-affected pRBCs. (A) High doses of mefloquine were unable to fully prevent some pRBCs from reaching the schizont stage, unlike artesunate. (B) More donor pRBCs (Gen_0) persisted in host circulation after treatment with a high dose of mefloquine compared with a high dose of artesunate. (C) Fitting the rate of decline of Gen_0 pRBCs in mice after treatment with mefloquine provides an upper bound estimate on the rate of removal of mefloquine-affected pRBCs. This estimated clearance is significantly slower than the rate of removal of artesunate-affected pRBCs.

where artesunate monotherapy exhibited high rates of recrudescence, and raises the idea that at least a subpopulation of parasites remains viable (although perhaps impaired) and capable of replication even after a single high dose of artesunate (Giao et al., 2001; Borrmann et al., 2003; Teuscher et al., 2010). This may be through mechanisms such as dormancy or slower maturation (Teuscher

et al., 2010; Codd et al., 2011; LaCrue et al., 2011; Chen et al., 2014; Dogovski et al., 2015), but this cannot be distinguished from the possibility that a proportion of parasites were completely unaffected by treatment from our study. As others have noted, the survival of a subpopulation could potentially lead to the rapid emergence of drug resistance to artemisinin monotherapy (Cheng et al., 2012). The clinical use of combination therapy both improves cure rates and delays the development of parasite resistance to the artemisinins (White, 1998, 1999; Giao et al., 2001; Adjuik et al., 2004).

The rapid onset of action of artesunate to halt parasite maturation is followed by rapid clearance of affected cells. By contrast, we observed both ongoing parasite maturation and parasite replication over the first 24 h after MQ treatment, and treated Gen_0 parasites were removed more slowly than with artesunate. These differences are consistent with the known ability of artesunate to rapidly reduce parasitemia in patients compared with other antimalarial drugs (Jiang et al., 1982), and its clinical effectiveness (Dondorp et al., 2005, 2010; Khoury et al., 2016).

The arrest of parasite maturation after artesunate treatment allowed us to examine in isolation the process of host clearance of different parasite life stages. Previous ex vivo spleen perfusion studies suggested that the spleen removes schizonts more effectively that younger forms, with an approximately 50% shorter half-life than rings (Safeukui et al., 2008). This was comparable to our in vivo estimates in which we observed a 33% shorter clearance half-life for schizonts compared with early-stage parasites. Interestingly, it appeared that phagocytic cells played a critical role in the removal of drug-affected young-stage parasites, and a less prominent role in the removal of schizonts. This is consistent with the view that artesunate-affected ring-stage parasites are usually removed actively from circulation via 'pitting' (Chotivanich et al., 2000, 2002; Buffet et al., 2006), a mechanism in which the affected parasite is removed from with the RBC and the cell is returned to circulation. This process is likely to involve phagocytic cells (Buffet et al., 2006). A similar mechanism is not believed to operate for mature schizonts. Further, the reduced deformability of schizonts may cause these parasites to be retained in the spleen via more mechanical means (Herricks et al., 2012). A limitation in our approach is that clodronate liposome is a broadly acting agent, depleting many phagocytic cells including macrophages, dendritic cells, and granulocytes (Couper et al., 2007; Terkawi et al., 2016). In this study we have not explored the relative roles of each of these cell types in the reduced clearance of parasites, nor have we quantified the extent of the depletion of each cell type, with the exception of dendritic cells (Supplementary Fig. S3). Identifying the relative contribution of various cell types in clearing artesunate-affected pRBCs will require further immunological interventions.

Our observations raise an interesting question about the importance of clearance; are parasites removed by the host severely damaged in some way (i.e. dead, moribund, or damaged to an extent that proliferation could not occur even if they were not removed from circulation)? Or does the host remove many potentially viable parasites also? In the former case, the host removal of moribund parasites would represent a "clean-up" function, less related to controlling pathogen numbers. In the latter case, clearance of potentially viable parasites would remain critical for host control of infection. Future experiments using longer-term phagocyte depletion may determine the importance of host clearance for clean-up versus parasite control.

The differences between the actions of artesunate and MQ also provide interesting insights into their clinical effects. The ongoing maturation and infection over the first 24 h of MQ treatment might be expected from its slower onset of action and slower observed parasite clearance rate in clinical studies (Jiang et al., 1982). However, even when a single generation of treated parasites was considered, it was unexpected that drug-affected parasites would be removed at such different rates for the two drugs. This raises the concept that different drugs may influence the subsequent rates of host removal of pRBCs. Thus, for example, if artesunate treatment leads to more free radical damage, then 'dead' (or incapacitated) parasites may be removed more quickly than cells affected by MQ.

In severe infection, the rate at which parasites become incapacitated is likely a crucial parameter of drug efficacy, minimising both sequestration and rupture. By contrast, the subsequent rate at which incapacitated cells are removed may be less important. In comparing MQ and artesunate, rapid incapacity and rapid clearance appear associated, but it is not clear that this need be the case for all drugs. Hence, separate measures of the rate of onset of drug action (the 'lag phase' (Flegg et al., 2011; Khoury et al., 2016)) and subsequent clearance of parasites may be helpful in evaluating new drug regimes.

Our adoptive transfer system provides a powerful tool for dissecting host-parasite interactions in vivo. However, it is important to note that there may be a number of differences in host and parasite physiology that may alter the effects of treatment. One notable example is the rate of 'clearance' of total parasitemia in our artesunate-treated mice, which was slower than that typically reported in humans (Abdulla et al., 2015). Notably, this clearance rate seemed similar to that observed in *Plasmodium falciparum* infection in humanised mice (Jiménez-Díaz et al., 2014), suggesting that it may be a host-specific effect. Hence some caution is required in the interpretation of our study in relation to human infections. However, despite this known limitation of the murine model, mice have provided a useful preclinical tool for assessing drug efficacy both historically and in recent times (Chawira et al., 1987; Rottmann et al., 2010; Jiménez-Díaz et al., 2014). Further, others have demonstrated that rate of decline in parasitemia after treatment with an array of drugs (although consistently slower in mice), is strongly correlated with the rate of decline of parasitemia in humans after treatment with those same antimalarial drugs (Jiménez-Díaz et al., 2013). As the first known direct in vivo dissection of the mechanisms of action for antimalarial drugs, we believe this study provides a useful framework for understanding drug action, which could be used in the future to examine other in vivo factors that might influence parasite control including pre-existing immunity (Looareesuwan et al., 1993).

Acknowledgements

This work was supported by the Australian Research Council (grant DP120100064), the National Health and Medical Research Council (NH&MRC), Australia (grants 1082022 (to MPD, DC, AH), 1080001 (to MPD), 1028634 (to AH) and 1028641 (to AH)), and the Australian Centre for Immunotherapy and Vaccine Development. The Australian Federal Government provided Australian Postgraduate Awards to DSK and KRJ, and the University of Queensland, Australia, provided International Postgraduate Research Scholarships to IS and JA, and a UQ Advantage Grant to IS. MSFS and SEB were supported by Australian NH&MRC Project Grants 613702 and 1028641. The authors do not have commercial or other associations that might pose a conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2017.05.009.

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