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Basigin is a druggable target for host-oriented antimalarial interventions

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Plasmodium falciparum is the parasite responsible for the most lethal form of malaria, an infectious disease that causes a large proportion of childhood deaths and poses a significant barrier to socioeconomic development in many countries. Although antimalarial drugs exist, the repeated emergence and spread of drug-resistant parasites limit their useful lifespan. An alternative strategy that could limit the evolution of drug-resistant parasites is to target host factors that are essential and universally required for parasite growth. Host-targeted therapeutics have been successfully applied in other infectious diseases but have never been attempted for malaria. Here, we report the development of a recombinant chimeric antibody (Ab-1) against basigin, an erythrocyte receptor necessary for parasite invasion as a putative antimalarial therapeutic. Ab-1 inhibited the PfRH5-basigin interaction and potently blocked erythrocyte invasion by all parasite strains tested. Importantly, Ab-1 rapidly cleared an established *P. falciparum* blood-stage infection with no overt toxicity in an in vivo infection model. Collectively, our data demonstrate that antibodies or other therapeutics targeting host basigin could be an effective treatment for patients infected with multi-drug resistant *P. falciparum*.

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Abbreviations used: AVEIXS, AVidity-based Extracellular Interaction Screen; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine aminopterin thymidine; HBS/T, Hepes-buffered saline/Tween-20; HIV, human immunodeficiency virus; NSG mouse, NOD scid gamma mouse; RH5, reticulocyte-binding homologue 5; V_H, antibody variable heavy chain; V_L, antibody variable light chain.

The repeated loss of antimalarial drugs as a result of the emergence of drug-resistant parasites requires continuing investment in drug discovery programs to ensure a future supply of treatments. Parasite drug resistance is a constant threat to efforts aimed at improving global health and is one of the major barriers for malaria elimination. An alternative strategy is to identify and target restrictive host factors that are less likely to be circumvented by resistance mutations in the rapidly evolving parasite genome (Prussia et al., 2011).

Host-oriented intervention strategies have been successfully applied for the treatment of several infectious diseases, particularly for HIV/AIDS. For example, ibalizumab, a humanized monoclonal antibody against human CD4—the receptor required for HIV entry in human T-helper cells—demonstrated high anti-HIV efficacy and was well tolerated by subjects (Bruno and Jacobson, 2010). Similarly, a humanized

anti-CCR5 antibody (PRO 140) has been shown to be safe for administration with potent and prolonged antiretroviral activity (Jacobson et al., 2010). Importantly, the CCR5 antagonist maraviroc has recently been approved by the US Food and Drug Administration for the treatment of HIV-infected patients (Lieberman-Blum et al., 2008). Other examples of host-oriented approaches for treating infectious disease are those proposed against poxvirus (Reeves et al., 2005), West Nile virus (Hirsch et al., 2005), and influenza virus (Morita et al., 2013). The concept of targeting host factors has also been considered for treating malaria (Prudêncio and Mota, 2013), but to our knowledge no host-oriented antimalarial therapeutic has been developed to date.

We previously identified basigin as an erythrocyte receptor for the parasite PfRH5 ligand,

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which is essential for invasion by all tested strains of *Plasmodium falciparum* (Crosnier et al., 2011). The crystal structure of *Pf*RH5 in complex with basigin has recently been solved (Wright et al., 2014), and *Pf*RH5 was found to interact with other parasite proteins (Chen et al., 2011; Reddy et al., 2015). In addition, *Pf*RH5 was shown to be efficacious against a heterologous strain blood-stage *P. falciparum* infection in a non-human primate infection model (Douglas et al., 2015), and the *Pf*RH5–basigin interaction has been implicated in host tropism (Wanaguru et al., 2013). Naturally acquired antibodies specific for *Pf*RH5 are associated with protection from clinical malaria (Tran et al., 2014; Chiu et al., 2014) and both anti-*Pf*RH5 and anti-basigin mAbs that block the basigin–*Pf*RH5 interaction in vitro can inhibit erythrocyte invasion by all tested parasite strains (Crosnier et al., 2011; Douglas et al., 2014; Ord et al., 2014). Interestingly, anti-basigin mAbs are much more potent than those binding to *Pf*RH5 ($IC_{50} \leq 1 \mu\text{g/ml}$ compared with 15 to $\sim 100 \mu\text{g/ml}$; Crosnier et al., 2011; Douglas et al., 2014; Ord et al., 2014), making basigin a particularly attractive antimalarial drug target. These differences in efficacy are likely due to the accessibility of basigin on the erythrocyte surface compared with the fleeting exposure of *Pf*RH5, which is sequestered within antibody-inaccessible intracellular organelles until required for invasion. While targeting a host protein to treat infectious diseases raises concerns about toxicity, therapeutic anti-basigin mAbs have been

successfully trialed in the clinic for treating both cancer (Chen et al., 2006; Xu et al., 2007) and graft-versus-host disease (Takahashi et al., 1983; Heslop et al., 1995; Macmillan et al., 2007), and were well tolerated by patients (Takahashi et al., 1983; Heslop et al., 1995; Chen et al., 2006; Xu et al., 2007).

Encouraged by these findings, and based on the recent success of antibody-based therapeutics to treat Ebola virus infection (Qiu et al., 2014), we now report the development of a high-affinity recombinant chimeric anti-basigin-1 antibody (Ab-1), as a putative antimalarial therapeutic. Ab-1 prevented erythrocyte invasion in vitro by multiple *P. falciparum* parasite lines, and cleared blood stage parasites with no evident toxic side effects in a murine *P. falciparum* infection model.

RESULTS AND DISCUSSION

To select a high affinity anti-basigin mAb, we screened a panel of hybridoma lines generated by immunizing mice with the purified recombinant ectodomains of human basigin (Fig. 1 a). One hybridoma clone was selected for further study because it secreted a mAb that demonstrated high reactivity against basigin (Fig. 1 b). This parent mAb was first tested for its ability to block the *Pf*RH5–basigin interaction. We found that in the presence of this mAb, the in vitro binding of *Pf*RH5 to basigin was potently inhibited, at concentrations comparable to the high affinity anti-basigin monoclonal MEM-M6/6 which was previously shown to block the

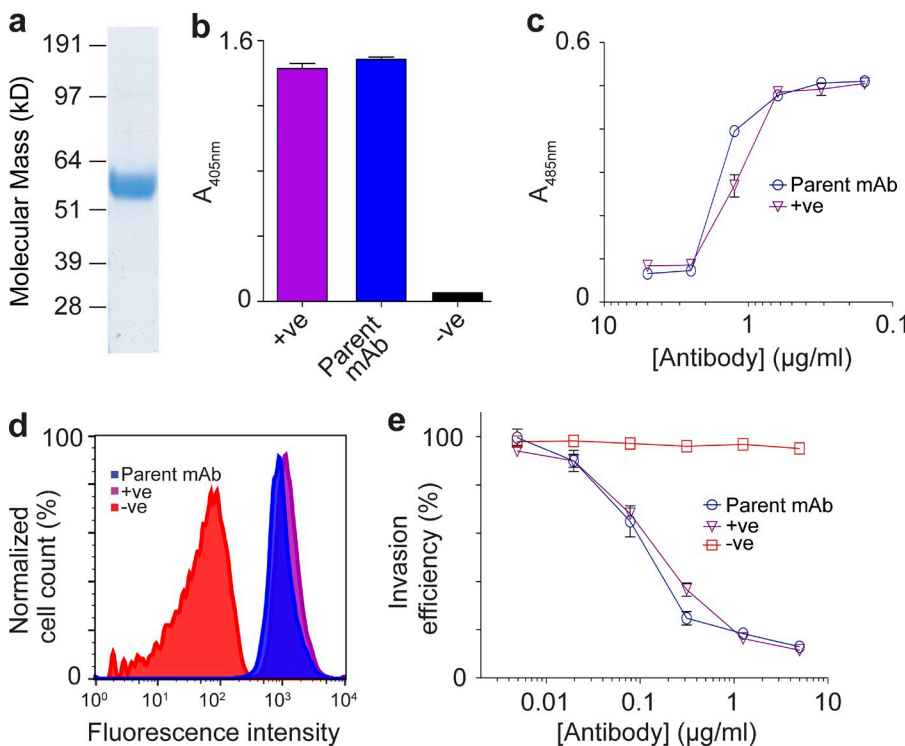


Figure 1. Development of an anti-basigin mAb which blocks *Pf*RH5 binding and *P. falciparum* erythrocyte invasion.

(a) Purified soluble recombinant basigin used to immunize mice was resolved by SDS-PAGE at the expected size (56 kD) and detected by Coomassie staining. (b) Analysis of the parent anti-basigin mAb binding to recombinant basigin by ELISA. Monomeric biotinylated basigin was immobilized in streptavidin-coated microtiter plates and probed using the parent anti-basigin mAb. Antibody binding is shown as an increase in absorbance at 405 nm. (c) The ability of the parent mAb to block the interaction between *Pf*RH5 and basigin was examined using the AVEXIS assay. Monomeric biotinylated *Pf*RH5 baits were immobilized in streptavidin-coated microtiter plates and direct interactions with basigin presented as a pentameric β -lactamase-tagged prey were quantified by hydrolysis of a β -lactamase colorimetric substrate producing a product that absorbs at 485 nm. A serial dilution of the anti-basigin antibody was preincubated with the basigin prey before presentation to the immobilized *Pf*RH5 bait. (d) Analysis of the parent mAb binding to basigin at the surface of human erythrocytes by flow cytometry.

The histogram shows the normalized cell counts as a function of fluorescence intensity. (e) Dose-dependent inhibition of *P. falciparum* (strain 3D7) erythrocyte invasion by the parent anti-basigin mAb. In all panels, data points represent means \pm SEM. $n = 3$. For all panels, a representative experiment of three replicates using independent samples is shown. Positive control (+ve) is the anti-basigin mAb MEM-M6/6 and negative control (-ve) is a mouse IgG.

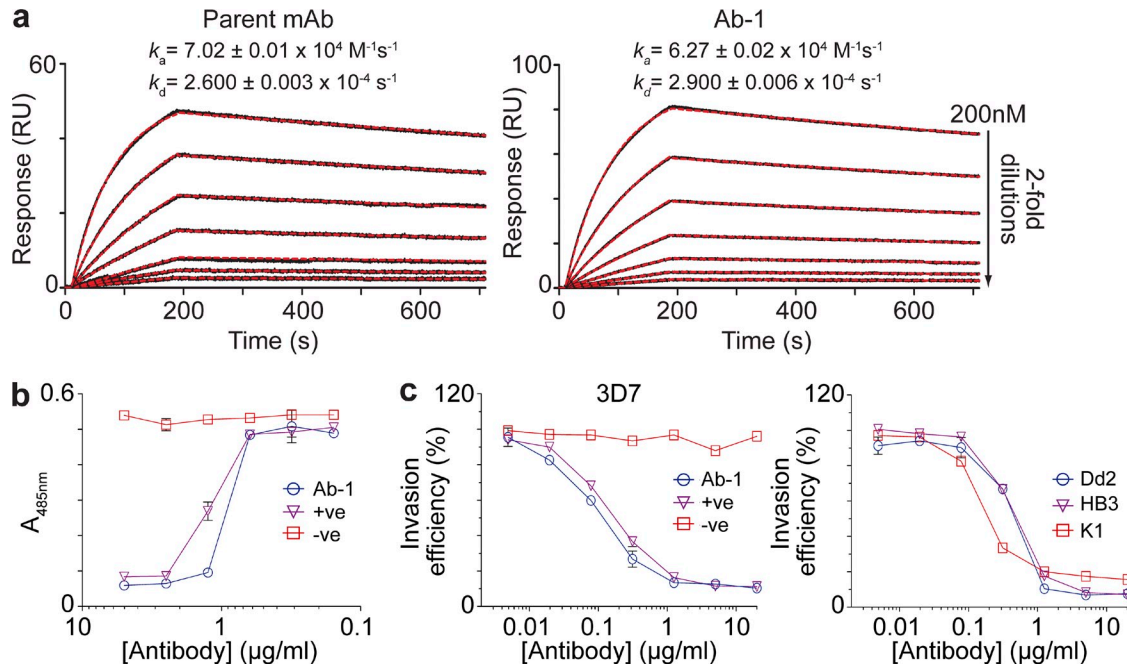


Figure 2. Ab-1 binds basigin with high affinity, blocks the *PfrH5*–Basigin interaction in vitro, and inhibits erythrocyte invasion by multiple *P. falciparum* strains. (a) The basigin-binding affinity of the parent mAb and Ab-1 were compared by using surface plasmon resonance. The monomeric equilibrium binding constant (K_d) for both parent mAb and Ab-1 were found to be ~ 4 nM. RU, response units. (b) Quantification of the ability of Ab-1 to block the interaction between *PfrH5* and basigin using the AVEXIS assay. Monomeric biotinylated *PfrH5* baits were immobilized in streptavidin-coated microtiter plates, and direct interactions detected using a pentameric β -lactamase–tagged basigin prey, as above. A serial dilution of the anti-basigin Ab-1 antibody was preincubated with the basigin prey before presentation to the immobilized *PfrH5* bait. (c) Quantification of Ab-1's ability to inhibit *P. falciparum* erythrocyte invasion in a parasite growth inhibition assay. Invasion of human erythrocytes by four *P. falciparum* strains (left, 3D7; right, K1, Dd2, HB3) in the presence of a dilution series of Ab-1. In panels b and c, the anti-basigin monoclonal antibody MEM-M6/6 and an isotype-matched anti-antibody were used as positive (+ve) and negative (-ve) controls, respectively. Data points show means \pm SEM. $n = 3$. For all panels, a representative experiments of three replicates using independent samples are shown.

PfrH5–basigin interaction (Koch et al., 1999; Crosnier et al., 2011; Fig. 1 c).

To determine whether the parent mAb could bind native basigin, we stained human erythrocytes and analyzed them by flow cytometry. We observed that the parent mAb stained erythrocytes essentially indistinguishably from MEM-M6/6, demonstrating that it is able to bind basigin expressed on the surface of human erythrocytes (Fig. 1 d). The efficacy of the parent mAb to prevent erythrocyte invasion was tested using an in vitro *P. falciparum* growth inhibition assay and was found to block erythrocyte invasion in a concentration-dependent manner (Fig. 1 e) with a similar IC_{50} to MEM-M6/6, which was previously shown to block invasion by all tested *P. falciparum* strains (Crosnier et al., 2011). These data establish that the parent anti-basigin mAb could potentially prevent erythrocyte invasion in vitro by inhibiting the *PfrH5*–basigin interaction and was therefore selected for further characterization.

To reduce the possibility of human anti–mouse antibody responses that might be expected in patients upon treatment, a chimeric antibody was developed by amplifying and sequencing the rearranged V_H and V_L antibody sequences (available from GenBank under accession nos. KP771818 and KP771819), which were then cloned into plasmids encoding the human

IgG₁ and constant κ chains, respectively. Co-transfecting HEK293 cells with both plasmids resulted in high levels of recombinant chimeric antibody (Ab-1). We mapped the Ab-1 epitope to the first of the two Ig-like domains of basigin by testing Ab-1 binding to each of the domains expressed individually (unpublished data). By using surface plasmon resonance, we demonstrated that Ab-1 bound basigin with virtually indistinguishable biophysical binding parameters compared with the parent mAb (Fig. 2 a). Ab-1 retained the ability to both block the *PfrH5*–basigin interaction and potentially inhibit the invasion of four culture-adapted *P. falciparum* strains from a range of geographical locations at low concentrations ($IC_{50} \sim 0.3$ μ g/ml; Fig. 2, b and c).

Mechanistically, we anticipated that Ab-1 would function by blocking the *PfrH5*–basigin interaction; therefore, to prevent the unnecessary and undesirable recruitment of antibody effector functions on erythrocyte cell surface, the human constant heavy chains of Ab-1 contained an established set of mutations (G1 Δ naB; Morgan et al., 1995; Ghevaert et al., 2013) that inhibit complement and Fc γ -receptor binding. We confirmed that these mutations were effective by demonstrating that Ab-1 binding to both C1q and Fc γ RIIA_{131His} was significantly reduced (unpublished data).

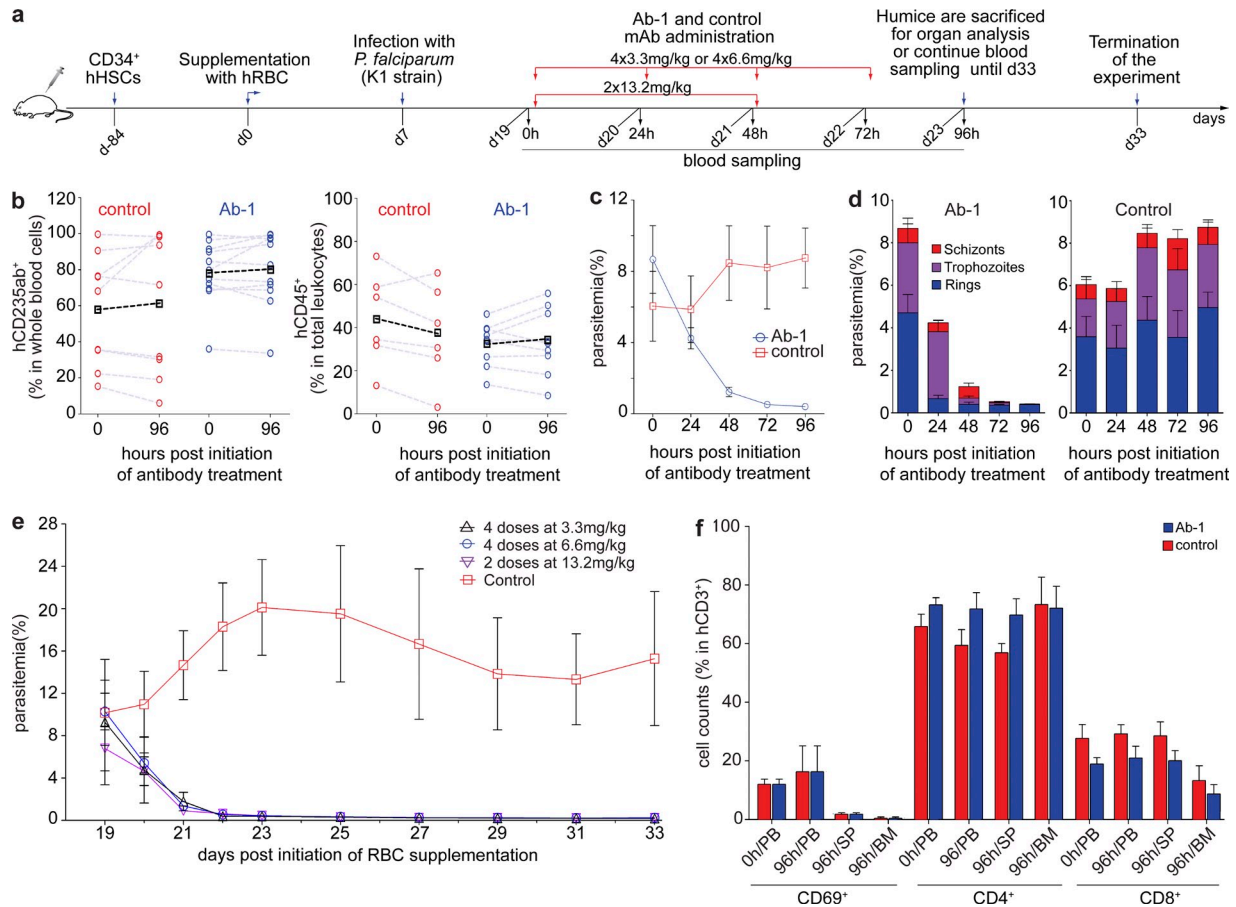


Figure 3. Ab-1 clears an established in vivo *P. falciparum* infection by blocking erythrocyte invasion in humanized mice, without observable toxicity. (a) Schedule of the in vivo experiments using humanized mice. (b) Percentages of circulating human (h) CD235ab⁺ (left) and hCD45⁺ (right) cells in humice before (0 h) and after 96 h of Ab-1 administration. The percentages of hCD235ab⁺ and hCD45⁺ cells in individual mice (open circles) before and after Ab-1 treatment are indicated by connecting lines. Means for each group are represented by black squares. (c) Course of parasitemia in humice after Ab-1 treatment relative to a control antibody. Data points represent mean \pm SEM. $n = 15$ (Ab-1), $n = 11$ (control). (d) Selective loss of ring stage parasites after Ab-1 treatment relative to a control. Data points represent mean \pm SEM. $n = 15$ (Ab-1), $n = 11$ (control). (e) Assessment of recrudescence and titration of Ab-1 dosing. Humice received the indicated Ab-1 treatments, and parasitemia was then monitored until completion of the experiment at day 33. In all three Ab-1 administration regimens, parasitemia reached undetectable levels within 72 h, and no recrudescence was observed for the duration of the experiment. In control mice, pooled data from the 3.3 and 6.6 mg/kg antibody administration regimens are shown. Data points represent mean \pm SEM: for 3.3 mg/kg, $n = 4$ (Ab-1) and $n = 3$ (control); for 6.6 mg/kg, $n = 9$ (Ab-1) and $n = 3$ (control); for 13.2 mg/kg, $n = 5$ (Ab-1). (f) Quantification of human activated T cells (CD69⁺), T-helper cells (CD4⁺), and cytotoxic T cells (CD8⁺) in humice before and after Ab-1 and control mAb administration. Cells were either isolated from peripheral blood (PB) just before antibody administration at 0 h, or from peripheral blood, spleen (SP), and BM after the last antibody injection at 96 h. Cells were quantified by flow cytometry. Bars represent means \pm SEM. $n = 5$ for CD8⁺ and CD4⁺, and $n = 3$ for CD69⁺. In all experiments, control was an isotype matched antibody; for all panels, a representative experiment of two independent replicates is shown.

To assess Ab-1 efficacy in vivo, we used a humanized mouse model (humice) of *P. falciparum* blood stage infection (Chen et al., 2014) in which the mouse immune cells and erythrocytes have been largely replaced by their human counterparts. In brief, immunodeficient pups were sublethally irradiated and grafted with CD34⁺ human hematopoietic stem cells (Fig. 3 A). Humice that exhibited >10% human leukocyte reconstitution (Fig. 3 b) within the total leukocyte population were selected and further injected daily with human erythrocytes (Fig. 3, a and b). Humice with high percentages (>20%) of circulating human erythrocytes were infected with *P. falciparum* by injecting a blood-stage parasite culture,

and were shown to support cycles of parasite blood stage replication and invasion (Fig. 3, a and c–e). Administration of four doses of 6.6 mg/kg of Ab-1 to humice with well-established *P. falciparum* infections (>5% parasitemia) resulted in a marked reduction of parasites to essentially undetectable levels within 72 h (Fig. 3 c). Consistent with our in vitro data, this was caused by a reduction in the number of ring-stage parasites within the first 24 h after administration (Fig. 3 d), confirming that the mechanism of action is to inhibit erythrocyte invasion.

We next sought to reduce the antibody dose and, additionally, to check for recrudescence of the infection once the

antibody administration was completed. We repeated the parasite challenge experiment using reduced antibody administration regimens (Fig. 3 a) and monitored the humice for at least 10 d after the final antibody dose to observe any relapse in the infection. In all three antibody dosing regimens, parasitemia again decreased to undetectable levels within 72 h (Fig. 3 d) and remained below detectable levels until day 33 where the experiment was completed. Together, these data establish that fewer or lower doses of Ab-1 are capable of clearing parasitemia in vivo and do not lead to recrudescence (Fig. 3 e).

Although clinical treatments with anti-basigin antibodies are generally well tolerated by patients, some studies using anti-basigin antibodies with unmodified heavy chains reported a decrease in circulating activated, but not naive, T cell counts (Heslop et al., 1995; Koch et al., 1999; Deeg et al., 2001; Macmillan et al., 2007). We did not observe any changes in the numbers of CD8⁺, CD4⁺ or activated CD69⁺ T cells after treatment with Ab-1 (Fig. 3 f), probably due to the mutations introduced into Ab-1 designed to disable antibody effector functions. Overall, the human leukocyte compartment remained unaffected demonstrating no cytotoxicity or pathology due to Ab-1 administration (Fig. 3, b and f).

In conclusion, we have developed a chimeric anti-basigin mAb that could serve as a host-orientated antimalarial drug. Ab-1 can rapidly clear a high-level *P. falciparum* blood stage infection in an in vivo infection model with no evidence of overt toxicity, and is highly potent at modest concentrations, suggesting that a single administration would be sufficient to clear an established infection. Notably, the IC₅₀ of Ab-1 (~0.3 µg/ml) in erythrocyte invasion assay (Fig. 2 c) is at least 50-fold lower than the IC₅₀ of anti-*Pf*PRH5 antibodies (~15–100 µg/ml) with similar affinity (Douglas et al., 2014; Ord et al., 2014), suggesting that targeting basigin would require a much lower dosing than targeting *Pf*PRH5. We observed no adverse side-effects associated with Ab-1 administration in a mouse with a humanized hematopoietic system, consistent with the current use of anti-basigin therapeutics to treat non-infectious diseases such as cancer in the clinic.

The repeated emergence of parasite drug resistance raises the frightening prospect that a strain that is refractory to all known therapies will evolve, and so a drug targeting a host factor could fill a valuable treatment niche which is likely to have the advantage of being less susceptible to the development of resistance. Importantly, therapeutic antibody production is now well-established, removing any technical uncertainties in manufacturing the antibody for therapy; indeed, similar approaches are currently being considered for other infectious diseases, including the current Ebola outbreak in West Africa (Qiu et al., 2014).

MATERIALS AND METHODS

Hybridoma generation and antibody screening. Hybridomas were generated using the SP2/0 myeloma using standard procedures and plated over ten 96-well plates before addition of HAT selection medium 24 h after the fusion. Hybridoma supernatants were harvested after 10 d and screened

by ELISA against the biotinylated ectodomain of basigin captured on streptavidin-coated plates.

Cloning and expression of recombinant Ab-1. The sequences encoding for the Ab-1 rearranged variable heavy and light chains were amplified by RT-PCR from hybridoma cDNA using a set of degenerate primers with PfuUltra II Fusion HS DNA polymerase, cloned into pCR-Blunt II-TOPO vector (Life Technologies), and sequenced. Regions encoding the variable heavy and light chains were made by gene synthesis and cloned into plasmids that contained modified human IgG₁ and constant kappa chains, respectively. Recombinant Ab-1 was expressed by HEK293F cells and purified on protein G columns (GE Healthcare).

Surface plasmon resonance (SPR). SPR analysis was performed by using Biotin CAPture kit (GE Healthcare), according to the manufacturer's instructions. To measure interaction affinity rather than antibody avidity, purified chemically biotinylated antibodies were immobilized on the streptavidin-coated sensor chip and purified monomeric (gel filtered) recombinant basigin was injected at a high flow rate (100 µl/min) to minimize confounding rebinding effects. Results were analyzed by using the BIAevaluation software (GE Healthcare), and kinetic parameters were obtained by fitting a 1:1 binding model to the reference subtracted sensorgrams.

AVidity-based Extracellular Interaction Screen (AVEXIS). AVEXIS assay was performed as previously described (Bushell et al., 2008). In brief, recombinant biotinylated *Pf*PRH5 was immobilized on the preblocked streptavidin-coated 96-well plate at concentrations sufficient for complete saturation of the available binding surface. The plate was then washed three times in HBST before addition of normalized β-lactamase-tagged pentameric basigin (Crosnier et al., 2011), which was preincubated for 1 h with a dilution series of the appropriate antibody. 2 h later, the plate was washed three times in HBST and once in HBS. Nitrocefin (250 µg/ml; 60 µl per well) was added and developed for ~3 h, before absorbance was measured at 485 nm on a PHERAstar plus (BMG Labtech) plate reader. All steps were performed at room temperature.

C1q binding assay. The binding of C1q to Ab-1 was examined by using an ELISA based assay. 100 µl 1.5 µg/ml of Ab-1 or hIgG1 isotype (SouthernBiotech) were used to coat the binding surface of a 96-well maxisorb plate (NUNC) overnight at 4°C. The plate was then incubated with a dilution series of human C1q (Sigma-Aldrich) for 2 h, followed by 1-h serial incubations with goat anti-hC1q polyclonal (at dilution 1:1,000; Abcam) and rabbit anti-goat IgG conjugated with alkaline phosphatase (Abcam). The plate was finally washed three times in PBST and once in PBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; Sigma-Aldrich) at 1 mg/ml (100 µl/well). Absorbance was measured at 405 nm on a PHERAstar plus (BMG Labtech) plate reader after 10–15 min.

FcγRIIA_{131HIS} binding assay. The binding of FcγRIIA_{131HIS} (α-chain) to Ab-1 was assessed by using an ELISA-based assay. Biotinylated FcγRIIA_{131HIS} was immobilized on a preblocked (with HBST, 2%BSA) streptavidin-coated 96-well plate (NUNC) at concentrations sufficient for complete saturation of the available binding surface (as determined by ELISA). The plate was washed with HBST and incubated for 2 h at room temperature with a dilution series of Ab-1 or hIgG1 isotype (SouthernBiotech). The plate was then washed again three times in PBST, followed by 1-h incubation with a donkey anti-hIgG F(ab)₂ polyclonal conjugated to alkaline phosphatase (Abcam) before addition of *p*-nitrophenyl phosphate (Substrate 104; Sigma-Aldrich) at 1 mg/ml (100 µl/well). Absorbance was measured at 405 nm on a PHERAstar plus (BMG Labtech) plate reader after 10–15 min.

***P. falciparum* culture and invasion assays.** All *P. falciparum* parasite strains were routinely cultured in human O+ erythrocytes at 5 or 2.5% hematocrit in complete medium (supplemented with 10% vol/vol human serum or 0.5% wt/vol albumax) and synchronized by treatment with 5% wt/vol

D-sorbitol solution. Parasitized red blood cells were counted as SYBR Green I-positive cells by using flow cytometry and the data analyzed with FlowJo (Tree Star). The anti-basigin mAb MEM-M6/6 (AbD Serotech) and an isotype-matched mAb were used as positive and negative controls, respectively.

P. falciparum infection model. Humanized mice were generated from NSG mice purchased from The Jackson Laboratory and sublethally irradiated before being injected intracardially with 2×10^5 CD34⁺ cells purified from human fetal liver. After reconstitution had been assessed, mice were administered daily with human RBCs collected from healthy donors without history of malaria infection. Humanized mice were infected by intravenous injection of 5×10^7 synchronized ring stage *P. falciparum* parasites (K1 strain) that had been cultured in vitro in leukocyte-free human erythrocytes. Infected mice received a daily dose of 6.6 mg/kg Ab-1 or an isotype-matched control mAb by intravenous injection and the level of parasitemia quantified by Giemsa-stained blood smears.

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J.C. Rayner and G.J. Wright are named inventors on a patent application relating to the use of anti-basigin reagents for treating malaria. The authors declare no additional competing financial interests.

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