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The *Plasmodium falciparum* blood stages acquire factor H family proteins to evade destruction by human complement

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Summary

The acquisition of regulatory proteins is a means of blood-borne pathogens to avoid destruction by the human complement. We recently showed that the gametes of the human malaria parasite *Plasmodium falciparum* bind factor H (FH) from the blood meal of the mosquito vector to assure successful sexual reproduction, which takes places in the mosquito midgut. While these findings provided a first glimpse of a complex mechanism used by *Plasmodium* to control the host immune attack, it is hitherto not known, how the pathogenic blood stages of the malaria parasite evade destruction by the human complement. We now show that the human complement system represents a severe threat for the replicating blood stages, particularly for the reinvading merozoites, with complement factor C3b accumulating on the surfaces of the intraerythrocytic schizonts as well as of free merozoites. C3b accumulation initiates terminal complement complex formation, in consequence resulting in blood stage lysis. To inactivate C3b, the parasites bind FH as well as related proteins FHL-1 and CFHR-1 to their surface, and FH binding is trypsin-resistant. Schizonts acquire FH via two contact sites, which involve CCP modules 5 and 20. Blockage of FH-mediated protection via anti-FH antibodies results in significantly impaired blood stage replication, pointing to the plasmodial complement evasion machinery as a promising malaria vaccine target.

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Introduction

The human complement is a first line of defence against microbial infections, triggering targeted attack and ultimately killing the microbial intruder. Major features of complement include C3b-mediated cell opsonization for phagocytosis, immune cell recruitment mediated by the release of C3a and C5a and the formation of a terminal complement complex (TCC) to induce targeted lysis of the microbe.

While the classical and lectin pathways are initiated in response to bacterial molecular patterns or antigen–antibody immune complexes, the alternative complement pathway (ACP) is activated continuously at a low rate by spontaneous hydrolysis of complement factor C3, which can form a C3 convertase and then cleaves C3 to C3a and C3b. Factor C3b exposes a reactive thioester and attaches covalently to the surface of microbes, encouraged by its hydroxyl-rich composition (Fig. 1A). C3b binding to cell surfaces in consequence triggers the ACP amplification loop via the assembly of C3b with activated factor B to form the C3 and C5 convertases, eventually resulting in TCC formation (Rother *et al.*, 1998).

In order to prevent any damage of self-cells by complement, human cells use a variety of complement inhibitors, which either exhibit decay-acceleration and/or co-factor activity; thus, they either help disassembling the C3 and C5 convertase complexes or cleavage of C3b by factor I, or which prevent TCC assembly. Inhibitors include membrane-bound regulators like complement receptor 1 (CR-1), CD55 or CD59 and central fluid-phase regulators like C4-binding protein (C4BP) and factor H (FH). As the main regulator of the ACP, FH has an important role in discriminating between self and non-self surfaces. The protein comprises 20 complement control protein (CCP) modules, beta-sandwich domains containing about 60

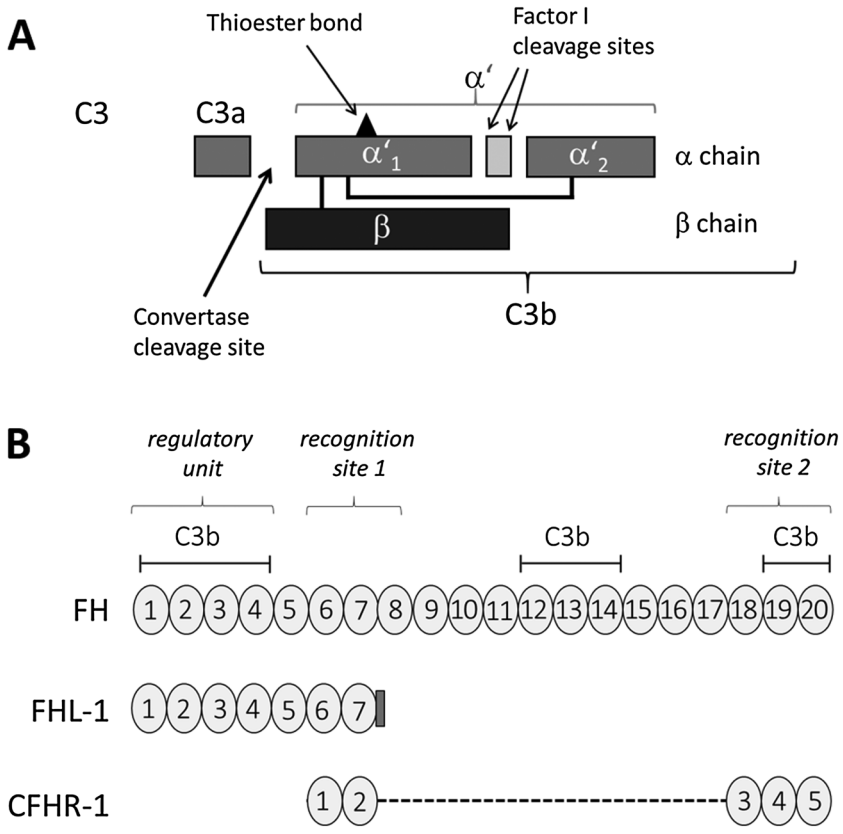

Fig. 1. Schematic depicting the domain structure of C3 and the CCP domains of FH, FHL-1 and CFHR-1.
A. Domain structure of C3 and cleavage sites [modified from Simon *et al.* (2013)]. The peptide sizes are the following: C3 (180 kDa), C3a (9 kDa), C3b α′ (109 kDa), β (75 kDa), α′ (101 kDa), α'_1 (67 kDa) and α'_2 (40 kDa).
B. Domain structures of FH, FHL-1 and CFHR-1. FH is composed of 20 CCP domains (CCP 1-20); FHL-1 is identical in sequence with the seven *N*-terminal CCP domains of FH (CCP 1-7) and includes at its C-terminal end an extension of four amino acids. CFHR-1 has five CCP domains homologous with CCP6-7 and CCP18-20 of FH. The regulatory unit, the recognition sites and the binding sites for C3b in FH, FHL-1 and CFHR-1 are indicated.

amino acid residues. Besides FH, the FH family consists of six more proteins, i.e. FH-like protein 1 (FHL-1), an alternative splicing product of FH comprising CCP modules 1–7, as well as the FH-related proteins CFHR-1 to CFHR-5, the genes of which originated from the FH gene via tandem duplication events (Fig. 1B) [reviewed in Józsi and Zipfel (2008), Skerka *et al.* (2013) and Józsi *et al.* (2015)].

Numerous microbial pathogens have evolved strategies to inhibit, control or prevent complement recognition, thus ensuring their survival in the human host. Such strategies include besides mimicking of host regulators and secretion of complement effector-targeting proteases, the recruitment of host regulators to the pathogens' surfaces [reviewed in Blom *et al.* (2009) and Zipfel *et al.* (2013)]. In the past complement evasion mechanisms have been described for microbial pathogens like *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitides*, *Borrelia burgdorferi* and *Candida albicans*. While the immune evasion mechanisms of these pathogens have been studied extensively [reviewed in Zipfel *et al.* (2007, 2008, 2013), Józsi and Zipfel (2008), Blom *et al.* (2009), Luo *et al.* (2013) and Zipfel and Skerka (2014)], they have only recently been described by us for the sexual stages of *Plasmodium* parasites, the causative agents of the tropical disease malaria (Simon *et al.*, 2013).

Malaria is a major global health burden, causing 198 million new cases and approximately 584 000 casualties annually worldwide (World Malaria Report, W.H.O, 2014). Malaria infections can persist for weeks or months in infected individuals, allowing the parasites to replicate in red blood cells (RBCs). Such compartmentalization within erythrocytes helps the parasites to evade recognition by the host immune system. Nonetheless, the parasites have to modify the RBC surface during growth in order to acquire nutrition from the blood plasma and to enable RBC sequestration on the blood vessel endothelium, making the infected erythrocyte assailable for the immune system. Further, the parasites have to leave the shelter of the RBC when the merozoites (MZs) explosively exit the

host cell to initiate another replication cycle. During the few minutes that the free MZ circulate until they infect another RBC, they can easily be recognized and destroyed by the immune components, including complement [reviewed in Wirth and Pradel (2012), Chan *et al.* (2014) and Wright and Rayner (2014)].

The complement system can be activated during malaria infection through different pathways [reviewed in Greenwood and Brueton (1974), Phanuphak *et al.* (1985), Nyakoe *et al.* (2009) and Biryukov and Stoute (2014)]. For example, it was shown that the parasite digestive vacuole, which during schizont rupture is released together with the MZs into the blood stream, activates the ACP (Dasari *et al*., 2012, 2014). Malaria infection-induced complement activation of the classical pathway via the formation of antigen–antibody immune complexes as well as activation of the mannose-binding lectin pathway have also been reported (Jhaveri *et al*., 1997; Adam *et al*., 1981; Klabunde *et al*., 2002; Garred *et al*., 2003; Stoute *et al*., 2003).

Another point of attack for the human immune system to kill malaria parasites is the mosquito midgut stages. The malaria parasites are taken up by the mosquito during the blood meal in form of sexual precursor cells, the intraerythrocytic gametocytes. Within minutes following their uptake, these emerge from the enveloping RBCs in response to stimuli present in the mosquito midgut and transform into fertile gametes. The emerging gametes are vulnerable to the factors of the blood meal, including antibodies and complement proteins [reviewed in Pradel (2007), Kuehn and Pradel (2010) and Wirth and Pradel (2012)].

In a recent study, we identified FH as the factor responsible for protecting the extracellular gametes of *Plasmodium falciparum* from ACP-mediated lysis (Simon *et al*., 2013). We showed that human complement is active in the mosquito midgut for 1 h after the blood meal and during this time represents a severe threat to the emerging gametes. In order to escape the ACP, gametes rapidly bind FH and FHL-1 from the blood meal, once they have egressed from the enveloping erythrocyte. Binding of FH to the parasite surface then promotes inactivation of surface-bound C3b via factor I.

We further identified the plasmodial transmembrane protein GAP50 (gliding-associated protein 50) as the FH-binding receptor of *P. falciparum* gametocytes. GAP50 was originally assigned to the parasite inner membrane complex (IMC), an alveolar double-membrane structure underneath the parasite plasmalemma, which mediates both cell motility and stability. Whereas the *C*-terminal transmembrane helix of GAP50 is anchored within the outer IMC membrane, the *N*-terminal portion protrudes into the IMC lumen (Baum *et al*., 2006; Sanders *et al*., 2007; Frénal *et al*., 2010; Bosch *et al*., 2012). Although not motile, the gametocytes of *P. falciparum* possess an IMC, which here functions to stabilize their crescent shapes (Dearnley *et al*., 2012; Kono *et al*., 2012; Simon *et al*., 2013). The IMC disintegrates during gametogenesis, and then GAP50 relocates from the IMC to the plasmalemma, where it binds FH via CCP5-7, thus protecting the extracellular gametes from attack by the human complement (Simon *et al*., 2013). In consequence, antibodies directed against GAP50 reduce FH binding to the gamete surface, leading to impaired gametogenesis and complement-mediated destruction of the parasite. In accordance with these findings, standard membrane feeding assays demonstrated that the functional blockade of either GAP50 or FH by antibodies inhibits transmission of the parasite from the human to the mosquito (Simon *et al*., 2013).

While these data give a first glimpse of the complex mechanisms the malaria parasites use to evade the human complement system, there is hitherto no published evidence of ACP evasion by the asexual blood stages (ABSs). We now demonstrate that the intraerythrocytic schizonts as well as the free MZ bind FH, FHL1 and CFHR-1 to their surfaces, thereby inactivating C3b to ensure their survival during the erythrocytic replication phase.

Results

Human complement affects the erythrocytic replication cycle of P. falciparum

The aim of this study was to investigate the protective effect of FH and FH family proteins on blood stage progeny of *P. falciparum*. We initially evaluated if the ABSs of *P. falciparum* are vulnerable to the human ACP during erythrocytic replication. ABSs with a starting parasitemia of 0.5% were cultivated in cell culture medium supplemented with 10 vol% of normal human serum (NHS) or heat-inactivated serum (HIS) at 37°C over a period of three generation cycles, and the parasitemia was determined every 12 h. Medium was exchanged every 12 h to ensure the presence of active complement throughout the experiments. During this period the viability of the malaria parasites was impaired when these were cultivated in NHS. The differences in parasitemia between NHS-treated and HIS-treated cultures increased from generation 1 to 3 and at the beginning of generation 4 (120 h following seeding), the parasitemia of NHS-treated parasite cultures was significantly reduced by 6% compared with the HIS-treated control (Fig. 2A). The major reductions in parasitemia occurred during the transitions between the sequential cycles, indicating that active complement either blocks or kills the MZs before invasion. The percentages of ring stages, trophozoites, immature and mature schizonts at each time point, however, did not differ between NHS-treated and HIS-treated ABS cultures during the 120-h cultivation period (Fig. S1).

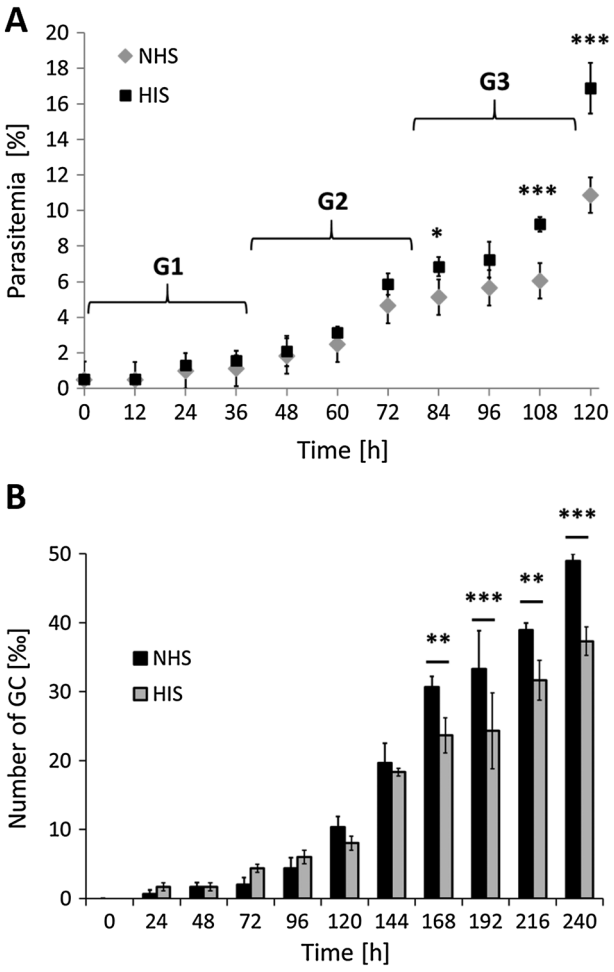

Fig. 2. Effect of human complement on ABS replication and gametocytogenesis.
A. Effect of human complement on ABS replication. Ring stage cultures of strain NF54 with a starting parasitemia of 0.5% were maintained in cell culture medium supplemented with either 10 vol% NHS or HIS at 37°C over a period of 0–120 h to follow three generations (G1–3). The parasitemia was evaluated every 12 h via Giemsa smears. Significant differences in parasitemia are indicated ($^{*}p < 0.05$, $^{***}p < 0.001$; Bonferroni post-test, two-way ANOVA). The experiment was performed in triplicate (mean ± SD); the data are representative for one of two independent experiments.
B. Effect of human complement on gametocytogenesis. Ring stage cultures of strain NF54 with a starting parasitemia of 5% were maintained in cell culture medium supplemented with either 10 vol% NHS or HIS at 37°C over a period of 0–240 h. The gametocytemia was evaluated every 24 h via Giemsa smears. Significant differences in gametocyte numbers are indicated ($^{**}p < 0.01$, $^{***}p < 0.001$; Bonferroni post-test, two-way ANOVA). The experiment was performed in triplicate (mean ± SD); the data are representative for one of two independent experiments.

To confirm that NHS used in the previously mentioned experiment was active, frozen pooled NHS samples were thawed and added to cell culture medium at 20 vol%. The medium was then kept at 37°C for different time periods, ranging from 30 min to 24 h, and was subsequently added to a fixed number of pig RBCs in RPMI medium at 37°C. After 1 h the absorbance of the supernatant was measured at $OD_{405\,nm}$ and compared with the supernatant of pig erythrocyte samples incubated in cell culture medium supplemented with HIS. Pig erythrocytes lysed by distilled water were used as a positive control. In NHS-treated pig erythrocyte samples taken at 30 min to 12 h, the absorbance was significantly increased compared with the HIS-treated erythrocyte samples (Fig. S2), demonstrating that the pig erythrocytes were partially lysed during this time period. The data indicate that NHS kept its complement-lytic activity for more than 12 h after thawing.

Because gametocytogenesis is induced in ABS parasites by stress factors like drug pressure or immune response molecules [reviewed in Talman *et al.* (2004), Alano (2007) and Pradel (2007)], we further investigated if NHS represents a stress factor that induces gametocyte formation. ABSs at a starting parasitemia of 5% were cultivated in cell culture medium either supplemented with 10 vol% NHS or HIS over a period of 240 h. Medium was exchanged every 12 h to ensure the presence of active complement throughout the experiments. While in both cultures, small amounts of gametocytes were detectable as early as 24 h after seeding, the numbers of gametocytes were significantly increased between 168 and 240 h after seeding in cultures treated with NHS (Fig. 2B). In NHS-treated cultures, furthermore, a higher percentage of stage V gametocytes were detectable compared with HIS-treated cultures (Fig. S3).

We then investigated if human complement causes deposition of C3b on the surface of infected RBCs (iRBCs), which can progress to TCC formation. As the decisive factor of the ACP, C3b continously forms because of the spontaneous hydrolysis of C3 into C3a and C3b and triggers complement activation leading to TCC formation by complement components C5b-9 at the surfaces of target cells. Indirect immunofluorescence assays (IFAs) using anti-C3 antibody demonstrated deposition of C3b on the surfaces of schizont-iRBCs when these were cultivated in the presence of NHS. The ABSs were counter-labelled with antibodies against the parasite plasmalemma-anchored MZ surface protein MSP1 (Holder and Freeman, 1984; Blackman *et al.*, 1991; reviewed in Kadekoppala and Holder, 2010). In order to distinguish between intact schizont-iRBCs and freed MZs, we either incubated the ABSs with 20 vol% NHS for 1 h and then fixed with paraformaldehyde before immunolabelling or the live schizont-iRBCs were treated with saponin to permeabilize the RBC and parasitophorous vacuole membranes and to gain access to the MZs before incubation with NHS for 1 h, followed by paraformaldehyde-fixation and immunolabelling. The IFAs revealed increased C3b labelling of intact schizont-iRBCs as compared with the C3b deposition on the surface of non-infected RBCs (niRBCs) (Fig. 3A). Noteworthy, a higher C3b labelling was also observed for freed MZs,

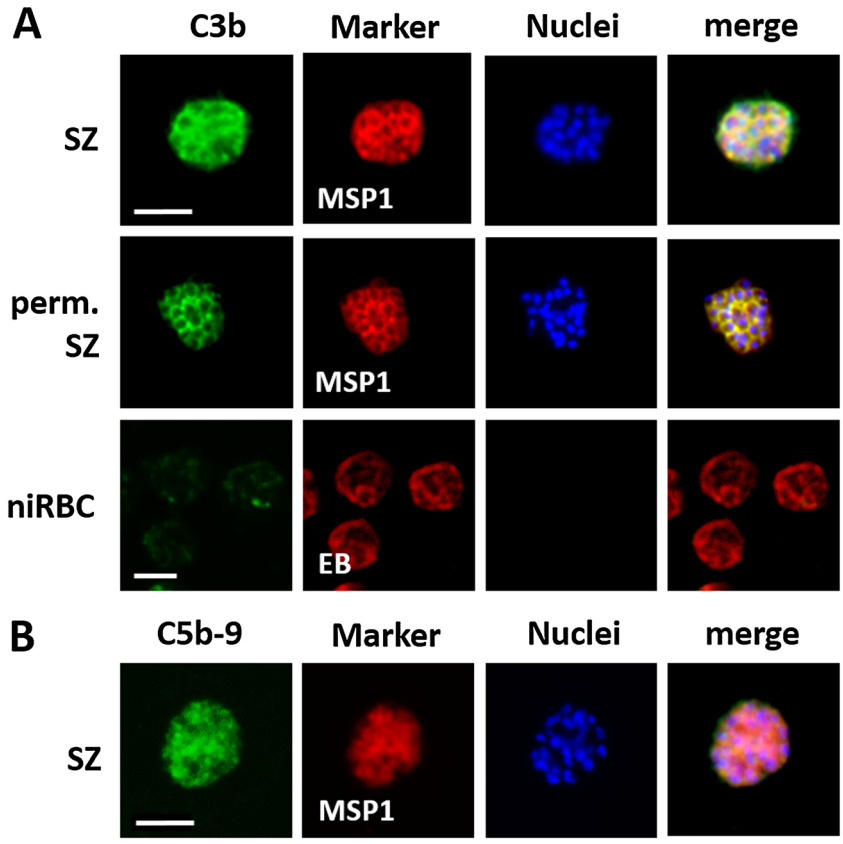

Fig. 3. Complement factor deposition on the surfaces of ABS parasites.
A. C3b deposition on the surfaces of ABS parasites. Intact schizonts (SZ), saponin-permeabilized (perm.) schizonts and niRBC as control were incubated with 20 vol% NHS for 1 h and subjected to IFAs. C3b deposition was demonstrated by immunolabelling with anti-C3 antibody (green). ABS parasites were detected by labelling with anti-MSP1 antibody; the niRBCs were highlighted by Evans Blue (EB) staining (red).
B. TCC formation on the surfaces of iRBCs. Intact schizonts were incubated with 20 vol% NHS for 1 h and subjected to IFAs. TCC formation was detected by labelling with mAb anti-C5b-9 (green). ABS parasites were detected by labelling with anti-MSP1 antibody (red). The nuclei were counterstained with Hoechst stain (blue). The data are representative for one of three independent experiments each. In the IFAs, strain F12 was used, similar results were observed for strain NF54. Bar, 5 μm.

demonstrating that C3b accumulates both on the surfaces of schizont-iRBCs and of MZs. No labelling was observed, when sera of non-immunized mice (NMS) was used as a negative control in the IFAs (Fig. S4A).

To verify that saponin-permeabilization of live schizont-iRBCs renders the MZs accessible to molecules like the complement factors, we performed a control experiment, in which schizont-iRBCs were treated with saponin before the cells were fixed with glutaraldehyde in suspension. Subsequently, an IFA was performed, using anti-MSP1 antibody to highlight the MZ plasmalemma, without any further permeabilization step during this process. The IFA resulted in dominant MSP1 labelling of MZs, when the schizont-iRBCs were permeabilized by saponin before fixation. When untreated schizont-iRBCs were fixed and used in the IFA as a negative control, no MSP1-labelling was observed (Fig. S4B).

Subsequently, lysates of schizont-iRBCs or niRBCs incubated with 20 vol% NHS for 1 h were subjected to gel electrophoresis, followed by Western blot analysis, and probed with anti-C3 antibodies. Purified C3b was loaded on the gel in order to demonstrate the unprocessed α' and β chains of C3b having mobilities of 101 and 75 kDa respectively (Fig. 1A). The Western blots demonstrated an increased labelling for the β chain in the schizont-iRBC lysate compared with niRBC lysate (Fig. 4A). Noteworthy, in the iRBC lysate, the band of the α' chain was very weak, while the α'_1 and α'_2 peptides with molecular weights of 67 and 40 kDa were visible. The presence of α'_1 and α'_2 demonstrates processing of α' in these samples, indicating that the iRBC-bound C3b is cleaved by factor I (Fig. 4A).

We further demonstrated the presence of TCCs on the surfaces of schizont-iRBCs cultivated in 20 vol% NHS via IFA, using a monoclonal antibody (mAb) directed against C5b-9 (Fig. 3B). The schizonts were counterlabelled with rabbit anti-MSP1 antibody. In total, 90 ± 1.2% of schizont-iRBCs (n = 100, in triplicate) stained positive for the C5b-9 complex. The TCCs were also detectable in lysates of schizont-iRBCs, when gel electrophoresis under non-reducing conditions followed by Western blotting with

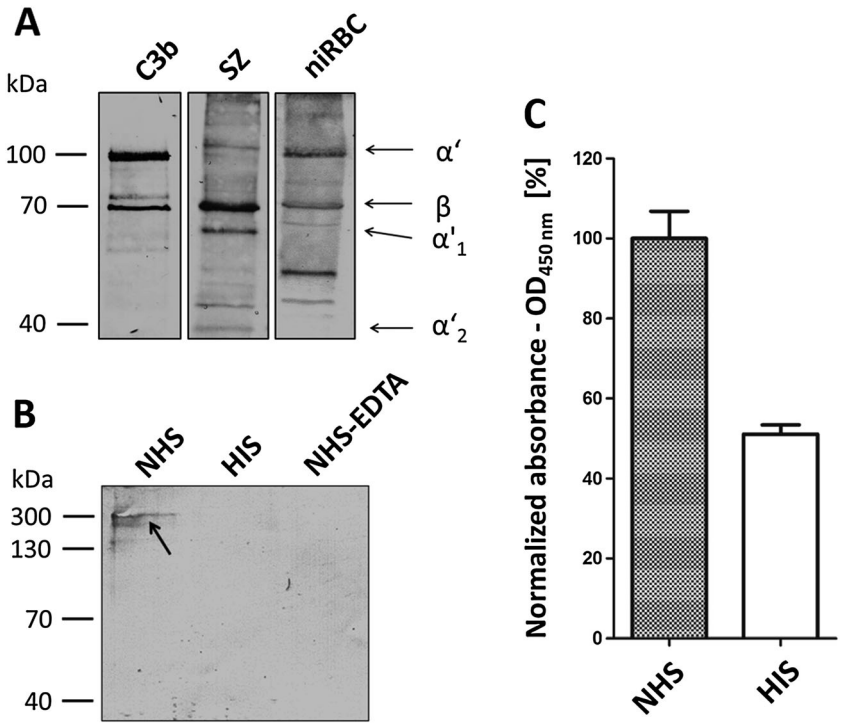

Fig. 4. C3b deposition and TCC formation in ABS parasites.
A. C3b deposition and processing on the surfaces of ABS parasites. SZ of strain F12 or niRBC were incubated with 20 vol% NHS for 1 h; lysates were prepared and subjected to gel electrophoresis followed by Western blotting with anti-C3 antibody. Purified C3b was loaded for positive control. Immunoblotting of the samples with anti-C3 antibody highlighted the unprocessed α′ and β chains (101 and 75 kDa respectively), as well as processed α'_1 and α'_2 (67 and 40 kDa respectively).
B. TCC detection in ABS parasites. Schizonts of strain F12 were incubated with 20 vol% NHS, HIS or EDTA-inactivated NHS; lysates were prepared and subjected to non-reducing gel electrophoresis followed by Western blotting with mAb anti-C5b-9 to detect the TCCs (approximately 330 kDa; arrow).
C. NHS-dependent TCC formation. Schizonts of strain F12 were incubated with 20 vol% NHS or HIS for 1 h and subjected to ELISA. Immunolabelling of the TCCs with mAb anti-C5b-9 was determined by measuring the relative absorbance at $OD_{450\ nm}$ (NHS set to 100%). The experiment was performed in triplicate (mean ± SD); the data are representative for one of two independent experiments.

anti-C5b-9 antibody was performed. In the blots, the TCC was running at a broad smear with an expected mobility of approximately 330 kDa (Fig. 4B). When the schizonts were cultured in HIS instead of NHS or when the NHS was inactivated by addition of 20 mM EDTA (Des Prez *et al*., 1975; Johnson *et al*., 2008), no TCC-positive band was detected in the schizont-iRBC lysates. TCC formation on the surfaces of schizont-iRBCs, cultivated either in 20 vol % NHS or HIS for 1 h, was further quantified via ELISA. The absorbance was twofold increased in samples of NHS-cultivated ABS cultures compared with the HIS control, when the mAb C5b-9 was used for immunodetection (Fig. 4C), indicating increased TCC formation on the iRBC surface in the presence of NHS.

Asexual blood stage parasites bind FH and FH family proteins on their surfaces

The previously mentioned experiments pointed to an inactivation of C3b deposited on the surfaces of schizont-iRBCs, which suggests an involvement of FH in complement evasion by the ABSs. To examine FH-mediated complement evasion in more detail, we initially performed IFAs to demonstrate FH binding to the surface of schizont-iRBCs. We either incubated the ABS parasites with 20 vol % NHS for 1 h before paraformaldehyde fixation, or we treated the live schizont-iRBCs with saponin to permeabilize the enveloping membranes in order to gain access to the MZs before NHS incubation for 1 h, followed by paraformaldehyde fixation and immunolabelling. The IFAs using anti-CCP1-20 antibody revealed binding of FH (and FH family proteins) both to the surfaces of intact schizont-iRBCs as well as to the freed MZs, which was increased compared with binding of FH to the surface of niRBCs (Fig. 5A). The binding of FH to schizont-iRBCs was quantified, and a total of 92 ± 3.5% of schizonts (n = 100, in triplicate) were FH-positive. For negative control, we labelled the paraformaldehyde-fixed ABSs with antibodies directed against C4BP, a regulator of the classical and lectin complement pathways, and no labelling was observed (Fig. S5). For positive control in this experiment, *Escherichia coli* were used in the IFAs and a strong immunolabelling for C4BP was detectable.

Subsequently, Western blot analyses were performed to investigate binding of FH and FH family proteins in more

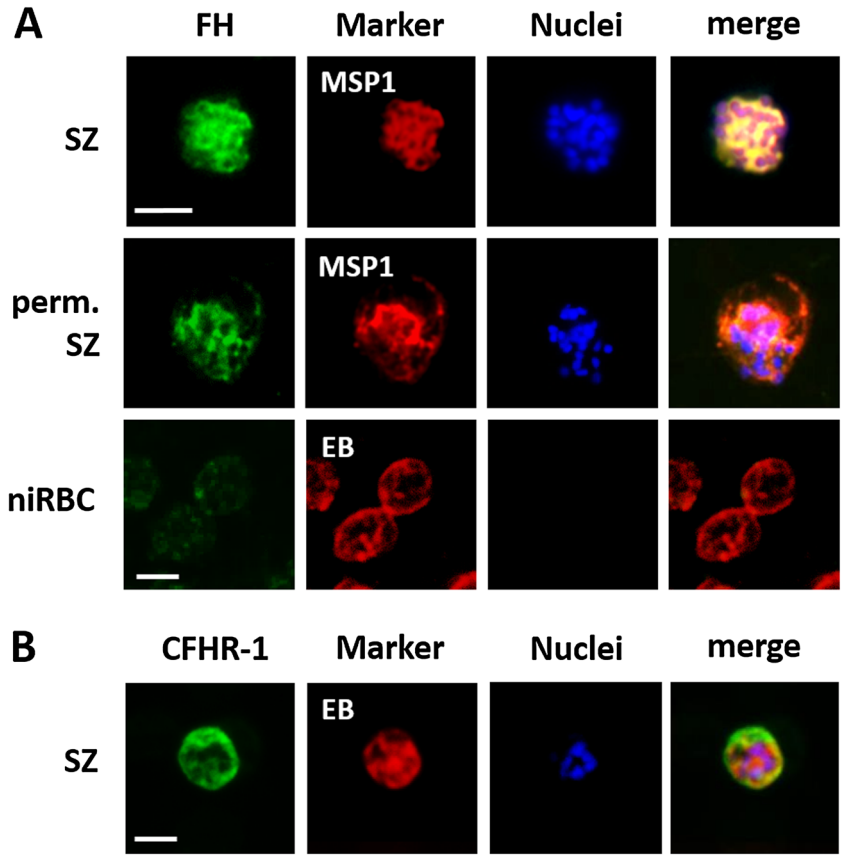

Fig. 5. Binding of FH and CFHR-1 to the surfaces of ABS parasites.
A. Binding of FH to the surfaces of ABS parasites. Intact SZ, saponin-permeabilized (perm.) schizonts and niRBCs as control were incubated with 20 vol% NHS for 1 h and subjected to IFAs. FH binding was demonstrated by immunolabelling with anti-CCP1-20 antibody (green). ABS parasites were detected by labelling with anti-MSP1 antibody; the niRBCs were highlighted by Evans Blue (EB) staining (red).
B. Binding of CHFR-1 to the surfaces of ABS parasites. Intact schizonts were incubated with 20 vol% NHS for 1 h and subjected to IFAs. CHFR-1 binding was detected by labelling with anti-CHFR-1 antibody (green). ABS parasites were detected by labelling with anti-MSP1 antibody (red). The nuclei were counterstained with Hoechst stain (blue). The data are representative for one of three independent experiments each. In the IFAs, strain F12 was used; similar results were observed for strain NF54. Bar, 5 μm.

detail. Schizont-iRBCs and niRBCs were incubated in 20 vol% NHS for 1 h, and lysates were prepared. Furthermore, MZs were isolated following a protocol by Boyle *et al.* (2010) before NHS incubation and lysate preparation. Lysates were subjected to non-reducing gel electrophoresis followed by Western blotting using anti-CCP1-20 antibody. Western blotting revealed that both the intact schizont-iRBCs as well as the isolated MZ bind FH and FHL-1, which migrated with the expected molecular weights of 155 and 37 kDa (Fig. 6A). Noteworthy, the schizont-iRBCs but not the niRBCs additionally bound CFHR-1; the α and β variants of CFHR-1 migrated with the expected molecular weights of 34 and 36 kDa. NHS was used as a positive control in the Western blots. Quantification of band intensities between FH bound to schizont-iRBCs and niRBCs demonstrated a significantly increased binding of FH to the parasitized RBCs (Fig. 6B).

We investigated the CFHR-1 binding in more detail, using a specific anti-CFHR-1 antibody, which recognizes CFHR-1 and FH, but not FHL-1. In IFAs, a total of 96 ± 0.6% of schizont-iRBCs ($n = 100$, in triplicate) were positive for labelling with the anti-CFHR-1 antibody (Fig. 5B). Western blotting confirmed that schizont-iRBCs but not niRBCs bind CFHR-1, and the α and β variants of CFHR-1 were detected in the schizont-iRBC lysates (Fig. 6C).

We then aimed to determine which of the CCP modules of FH are involved in binding to the ABS parasites. Seven recombinant deletion mutants, comprising different CCP modules, were investigated for binding. Recombinant FH, comprising CCP1-20, was used as a positive control in the assays. Initially, we separated the purified fragments by gel electrophoresis in order to determine purity and molecular mobilities of the respective proteins (Fig. S6). Subsequently, schizont-iRBCs were incubated in cell culture medium supplemented with different recombinant FH deletion mutants (10 ng μl^{-1}). The schizont-iRBCs were thorougly washed, and the respective lysates were then subjected to non-reducing gel electrophoresis

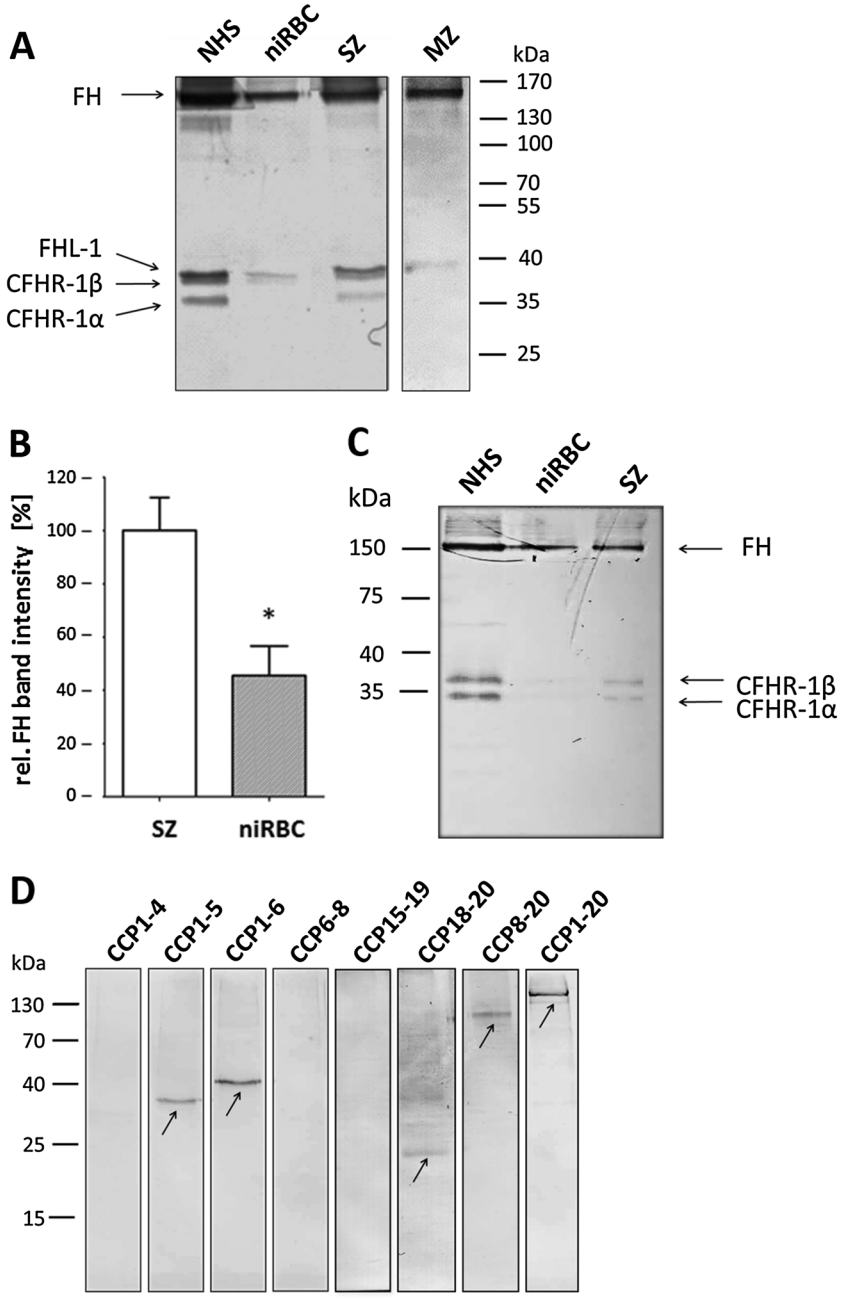

Fig. 6. Binding of FH and family proteins to ABS parasites.
A. Acquisition of FH and family proteins by ABS parasites. SZ and purified merozoites (MZ) of strain F12 or niRBCs for control were incubated with 20 vol% NHS for 1 h; lysates were prepared and subjected to non-reducing gel electrophoresis followed by Western blotting with anti-CCP1-20 antibody. NHS was loaded for positive control. Immunoblotting demonstrated FH (155 kDa), FHL-1 (37 kDa) and CFHR-1β (36 kDa) and CFHR-1α (34 kDa) in the samples. The data are representative for one of five independent experiments.
B. Quantification of FH-binding to ABS parasites. Relative band intensities for FH from lysates of schizonts or niRBCs after incubation with NHS were measured from five independent Western blot experiments (mean ± SD) using the imageJ programme (NHS set to 100%). Significant differences in intensities are indicated ($^{*}p < 0.05$; Student's *t*-test).
C. Binding of CFHR-1 to ABS parasites. SZ of strain F12 or niRBCs for control were incubated with 20 vol% NHS for 1 h; lysates were prepared and subjected to non-reducing gel electrophoresis followed by Western blotting with anti-CFHR antibody. NHS was loaded for positive control. Immunoblotting demonstrated CFHR-1β (36 kDa) and CFHR-1α (34 kDa) in the samples. The data are representative for one of two independent experiments.
D. Identification of CCP modules involved in FH-binding to ABS parasites. Schizonts of strain F12 were incubated in cell culture medium in the presence of the indicated recombinant FH deletion mutants at a concentration of 10 ng μl-1 before being subjected to non-reducing gel electrophoresis and Western blotting, using polyclonal anti-CCP1-20 antibody. Incubation with FH (CCP1-20) was used as a positive control. Arrows indicate the peptide bands. The data are representative for one of two independent experiments.

followed by Western blot analysis, using anti-CCP1-20 antisera, in order to detect the FH fragments. Western blotting demonstrated binding of the peptides CCP1-5, CCP1-6 CCP18-20, CCP8-20 and of full-length FH, CCP1-20, used as a positve control, while the FH delection mutants CCP1-4, CCP6-8 and CCP15-19 did not bind (Fig. 6D). Altogether, the data point to the involvement of two CCP modules, CCP5 and CCP20, in binding of FH and FH family proteins to the schizont-iRBCs.

Because FH was particularly bound by the schizont-iRBCs, we aimed to investigate the potential correlation between FH binding and the formation of knobs on the erythrocyte surface in these stages. During schizont maturation, the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family proteins localize on knobs and bind endothelial cell surface molecules, like CD36, via their variable extracellular domains in order to adhere to the capillary walls and in consequence to escape clearance by the spleen [reviewed in e.g. Kirchgatter and Del Portillo (2005)]. Firstly, we investigated the percentile of knob-infected RBCs in the *P. falciparum* F12 strain used in our studies. Noteworthy, this laboratory strain has never not been selected by us for knob-formation. Knob-infected RBCs were identified using a previously published gelatin flotation purification method (Goodyer *et al.*, 1994) and resulted in roughly 42% of the schizont-iRBCs showing a gelatin-flotation behaviour known for knob-infected erythrocytes (Fig. S7A). We further studied the efficiency of the schizont-iRBCs to bind to recombinant CD36 via a microplate adhesion assay (Prudhomme and Sherman, 1999) using the Malstat reaction as a read-out for parasite abundance. The absorbance was approximately fourfold increased in samples of schizont-iRBCs bound to CD36-coated wells as compared with niRBC samples (Fig. S7B). Non-coated wells were used for negative control. Because the PfEMP1-based knobs are sensitive to treatment with trypsin (Leech *et al.*, 1984), we lastly investigated the FH-binding behaviour of schizont-iRBCs with and without prior trypsin treatment. Quantitative Western blot analysis demonstrated that treatment of the schizont-iRBCs with trypsin prior to incubation with 20 vol% NHS has no effect on binding of FH by these cells (Figs 7 and S8A). For control, niRBCs with or without trypsin-treatment were used, and here a significantly lower FH binding compared with the schizont-iRBCs was observed, confirming our earlier observations. The activity of trypsin was tested by demonstrating the cleavage of glycophorin A from the schizont-iRBC surface (Fig. S8B). In conclusion we postulate that binding of FH by the schizont-iRBCs is not mediated by knobs.

FH binding mediates C3b inactivation and protects the asexual blood stages from lysis

In a final set of experiments we aimed to investigate if FH binding protects the ABS parasites from complement-mediated destruction. A cofactor assay was employed to investigate if the surface-bound C3b is inactivated by FH and factor I. Schizont-iRBCs were cultivated in Albumax-complemented cell culture medium in the absence of human FH. Then purified FH was added to part of the cultures, and these were incubated with FH for 30 min. The parasite cultures were subsequently washed and incubated with purified C3b in the presence or absence of factor I for 30 min. Lysates were prepared and subjected to gel electrophoresis, followed by Western blotting, and probed with anti-C3 antisera. C3b was loaded as a positive control. In schizont-iRBCs, to which FH and factor I were added in order to inactivate C3b, the α' chain was processed by factor I, and the peptides α'_1 and α'_2 (67 and 40 kDa) appeared in addition to the protein bands of α' and β (101 and 75 kDa) (Fig. 8A). In samples without FH and/or factor I, however, the α' chain was not cleaved to α'_1 and α'_2. A minor degradation of C3b was observed in schizont-iRBC cultures supplemented with factor I, but lacking human FH, which might be due either to non-specific proteolysis or caused by remnants of bovine FH of the Albumax medium, which might have bound to the iRBCs (Fig. 8A).

To evaluate a potential role of FH in protection against the ACP-mediated growth impairment, schizont-iRBC cultures at a parasitemia of 2.5% were incubated with 20 vol% NHS in the presence of a mAb directed against FH modules CCP8-15 known to exhibit functional inhibition of the complement regulator (Oppermann *et al.*, 2006; Simon *et al.*, 2013) at a concentration of 100 μg ml^{-1}.

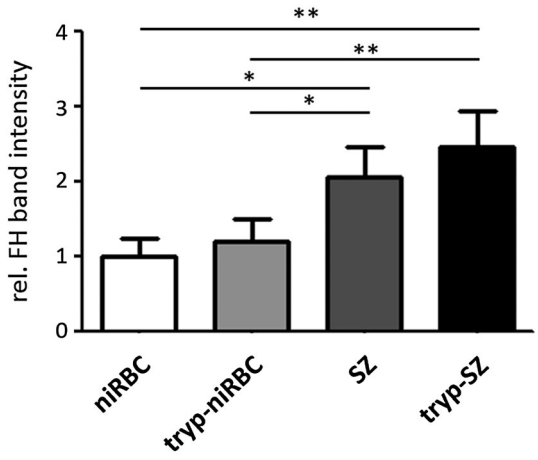

Fig. 7. Binding of FH by ABS parasites following trypsin-treatment. Schizonts of strain F12 were treated with 100 μg ml^{-1} trypsin for 30 min prior to incubation with 20 vol% NHS for 1 h. Lysates were prepared and subjected to non-reducing gel electrophoresis followed by quantitative Western blotting using anti-CCP1-20 antibody. Relative band intensities for FH from lysates of schizonts or niRBCs with and without trypsin-treatment were measured from five independent Western blot experiments (mean ± SD) using the imageJ programme (niRBC set to 1). Signficant differences in intensities are indicated ($^{*}p < 0.05$, $^{**}p < 0.01$; Student's *t*-test).

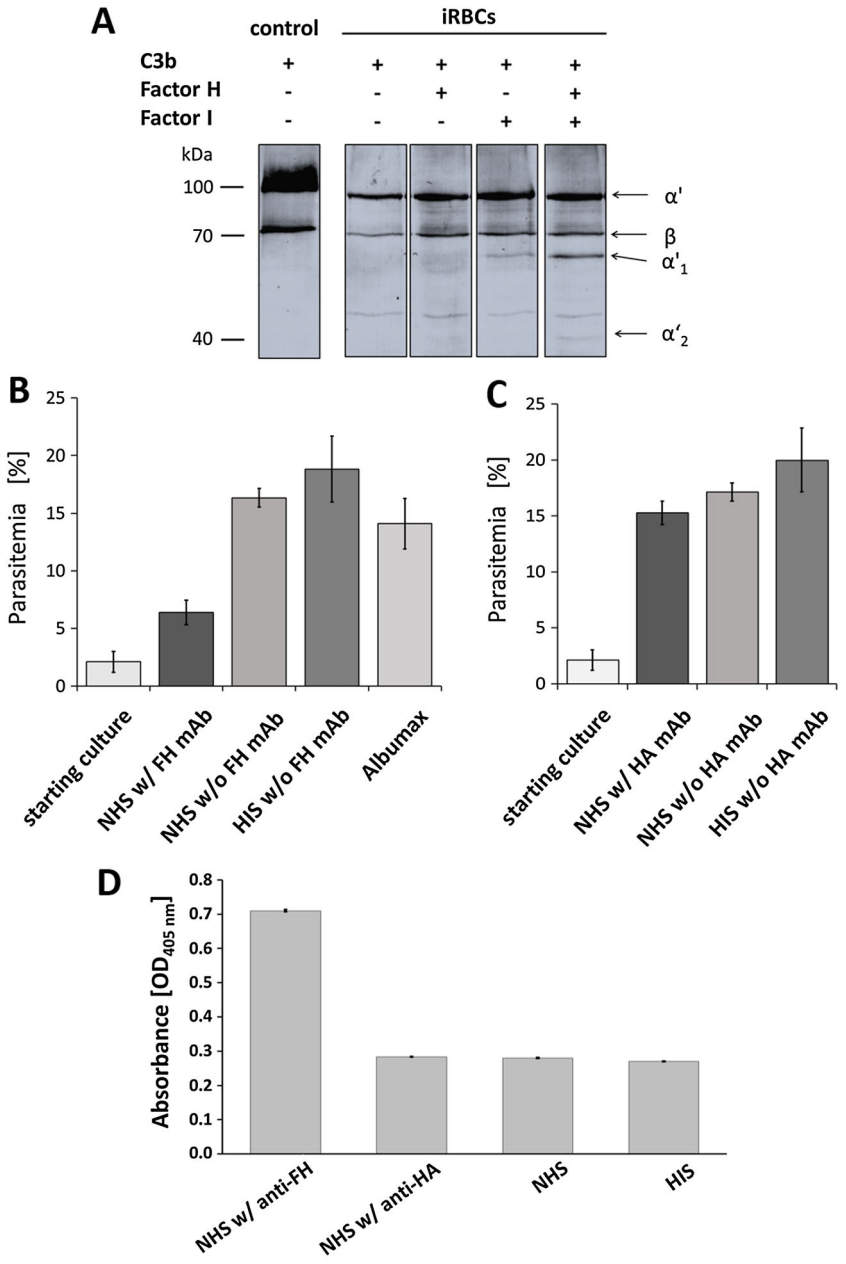

Fig. 8. Protection of ABS parasites from complement-mediated lysis by FH.
A. FH-mediated processing of C3b by factor I. Schizonts of strain F12 were incubated in cell culture medium in the presence or absence of purified FH for 30 min. The parasite cultures were subsequently washed and incubated with purified C3b in the presence or absence of factor I for 30 min. Lysates were prepared and subjected to gel electrophoresis followed by Western blotting and probed with anti-C3 antibodies to visualize schizont-bound C3b products α' (101 kDa) and β (75 kDa), and the processing products α'_1 (67 kDa) and α'_2 (40 kDa). C3b was loaded as a positive control. The data are representative for one of three independent experiments.
B. Effect of functional inhibition of FH on ABS growth. Schizont cultures of strain F12 with a starting parasitemia of 2.5% were maintained in cell culture medium supplemented with either 20 vol% NHS or HIS or in 10 vol% Albumax-supplemented medium in the presence or absence of mAb anti-FH over a period of 0–24 h, and the parasitemia was subsequently evaluated via Giemsa smears. The experiment was performed in triplicate (mean ± SD); the data are representative for one of two independent experiments.
C. ABS growth in the presence of anti-HA control antibody. Experiments were performed as described in B using a mAb against HA as negative control for B. The experiment was performed in triplicate (mean ± SD); the data are representative for one of two independent experiments.
D. Effect of functional inhibition of FH on iRBC lysis. Experiments were performed as described in B and C. The absorbance of the culture supernatants was measured at $OD_{405\ nm}$ to determine lysis of iRBCs.

Controls were incubated with NHS or HIS without mAb anti-FH or with Albumax-supplemented cell culture medium. At 24 h post-seeding, the parasitemia of the ABS cultures was decreased, when these were treated with the mAb anti-FH compared with controls, and a 10% reduction in parasitemia was measured compared with NHS-treated cultures lacking mAb anti-FH (Fig. 8B). When a mAb directed against hemagglutinin (HA)-tag was used as a negative control in the assays, no significant reduction of parasitemia was observed compared with the respective controls (Fig. 8C). Noteworthy, ABS cultures incubated with NHS in the presence of mAb anti-FH further showed a more than two-fold higher absorbance for haemoglobin at $OD_{405\,nm}$ compared with the controls lacking mAb anti-FH, indicating that here a higher number of iRBCs were lysed (Fig. 8D). In conclusion, the combined data show that FH protects the intraerythrocytic malaria parasites from lysis by the ACP by mediating inactivation of C3b on the surfaces of the iRBCs, an effect that can be derogated by antibody-mediated blockage of FH function.

Discussion

While the mechanism of FH recruitment as a means to evade destruction by human ACP is well known for bacterial pathogens like *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitides* or *Borrelia burgdorferi* [reviewed in Zipfel *et al.* (2007, 2008, 2013), Józsi and Zipfel (2008), Blom *et al.* (2009), Luo *et al.* (2013) and Zipfel and Skerka (2014)), the role of human complement during the infection with malaria parasites has only been investigated in detail within the past few years.

In a recent study, we identified FH as the factor responsible for protecting the extracellular gametes of *P. falciparum* from the human ACP, which is active in the mosquito midgut for 1 h after the blood meal and which during this time represents a severe threat to the emerging gametes. In order to escape the ACP, the gametes bind FH and FHL-1 from the blood meal, which then support inactivation of surface-bound C3b via factor I (Simon *et al.*, 2013).

In the same study, we identified the plasmodial transmembrane protein GAP50 as the FH-binding receptor. While in MZs and in non-activated intraerythrocytic gametocytes GAP50 localizes to the IMC, the protein relocates to the gamete plasmalemma upon activation (Simon *et al.*, 2013). During this time, the IMC disintegrates to leave the newly formed gametes defined by a single membrane (Sologub *et al.*, 2011). Consequently, antibodies directed against GAP50 are able to reduce FH binding to the gamete surface and inhibit parasite transmission from the human to the mosquito, making GAP50 a promising new candidate for transmission blocking vaccines (Simon *et al.*, 2013).

In accordance with our observations that the ACP is as a major threat for the malaria parasites in the mosquito midgut, transmission blocking antibodies against prominent sexual stage surface proteins like Pfs230 or Pfs25 require active human complement to kill the mosquito midgut stages, as was previously shown in standard membrane feeding assays (e.g. Read *et al.*, 1994; Williamson *et al.*, 1995; Healer *et al.*, 1997; Beiss *et al.*, 2015; Boes *et al.*, 2015; reviewed in Pradel, 2007). Furthermore, in the presence of anti-Pfs230 antibodies complement factor C1q binds to the gamete surface and activates the classical complement pathway (Simon *et al.*, 2013).

Until recently, antibodies directed against merozoite surface proteins, like MSP1, AMA-1 or EBA175, were thought to functionally inhibit RBC invasion by interfering with attachment and binding of the merozoite to its host cell (e.g. Hodder *et al.*, 2001; Miura *et al.*, 2009; Reiling *et al.*, 2012; Schwartz *et al.*, 2012). In a new study, however, Boyle *et al.* (2015) demonstrated that acquired invasion-inhibitory antibodies act through binding of C1q and activation of the classical complement pathway rather than by functionally inhibiting invasion. Without active complement, the majority of antibodies against merozoite surface proteins like MSP1 were not effective.

Interestingly, RBC invasion involves the human complement receptor CR-1, which is recognized by the reticulocyte-binding-like protein Rh4 located on the apical pole of the MZ (Spadafora *et al.*, 2010; Tham *et al.*, 2010). Approximately 1000 CR-1 receptors cover the RBC surface, recognize C3b-opsonized immune complexes and transport these to the liver-resident Kupffer cells for phagocytosis (Taylor *et al.*, 1997; van Lookeren Campagne *et al.*, 2007). The binding site of Rh4 was mapped to CCP1 of CR-1, and this region is known to be involved in binding C3b and C4b to accelerate decay of the C3 and C5 convertases (Park *et al.*, 2014). In consequence, Rh4 binding specifically inhibits the convertase decay-accelerating activity of CR-1 (Tham *et al.*, 2011).

Besides CR-1, RBCs also expose GPI-anchored complement regulators CD55 and CD59 on their surfaces, which promote acceleration of C3 convertase decay and TCC dysfunction respectively [reviewed in Zipfel and Skerka (2009)]. Both GPI-anchored proteins are abundantly present on the niRBC; however, they become partially internalized by malaria parasites following infection and can then be found bound to the parasitophorous vacuole membrane (Lauer *et al.*, 2000). Noteworthy, in this context, it was recently shown that the digestive vacuoles, when released during schizont rupture, activate the ACP, resulting among others in erythrophagocytosis of bystanders, particularly of iRBCs with reduced CD55 and CD59 levels (Dasari *et al.*, 2012, 2014).

It has previously been postulated that the expansion of MZ invasion ligands, resulting in multiple and apparently

redundant invasion pathways, and the presence of highly polymorphic merozoite surface proteins, as it is known for MSP1, are a means of immune evasion by the MZ [reviewed in Wright and Rayner (2014)]. We now show that besides this molecular distraction of the immune system through antigen diversity, the MZs also evolved a complement evasion mechanism by binding FH to their surfaces, resulting in C3b inactivation and in consequence in inhibition of TCC formation and parasite killing. Because we observed FH binding not only on the surface of the extracellular MZs but also on the schizont-iRBCs, our combined data provide evidence that both stages are highly vulnerable to human complement and thus need to acquire FH for protection.

Contrary to FH acquisition by the extracellular gametes, which is mediated via the binding of GAP50 to CCP5-7, the ABSs bind FH via CCP modules 5 and 20. Modules CCP18–20 represent a common binding site used by self-cells as well as by a variety of microbes like *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Candida albicans* or *Borrelia burgdoferi* to recruit host FH (see Fig. 1B; e.g. Oppermann *et al*., 2006; Józsi *et al*., 2007; Meri *et al*., 2013; reviewed in Zipfel *et al*., 2007, 2008, 2013; Józsi and Zipfel, 2008; Skerka and Zipfel, 2008; Ferreira *et al*., 2010). Interestingly, FH binding via CCP20 enhances C3b binding to FH and initiates the formation of a tripartite complex between FH, C3b and the microbial receptor, which then enhances the C3b-inactivating activity of FH (Meri *et al*., 2013). Binding of FH via the *N*-terminal portion (CCP1-7), on the other hand, yields the benefit for the pathogen to additionally recruit FHL-1 for complement evasion. Modules CCP6-7 can also be used by microbes like *Streptococcus pyogenes*, *Neisseria gonorrhoeae* or *N. meningitides* for complement evasion (e.g. Poltermann *et al*., 2007; Malito *et al*., 2013; reviewed in Zipfel *et al*., 2007).

Interestingly in this context, CCP7 plays an important role in autoimmune regulatory processes, and CCP7 mutations caused by single nucleotide polymorphisms can cause autoimmune defects such as age-related macular degeneration (AMD), a leading form of blindness. AMD is a chronic inflammatory disease associated with the replacement of tyrosine (Y) by histidine (H) at CCP7 position 402 [Y(402)H]. This exchange results in inappropriate ACP regulation in the retina, ultimately leading to the formation of drusen and vision loss (Skerka *et al*., 2007; reviewed in Skerka and Zipfel, 2008; Zipfel *et al*., 2010). Another FH polymorphism associated with AMD is the substitution of isoleucine with valine at position 62 [I(62)V], which results in and increased binding affinity for C3b and enhanced cofactor activity (Tortajada *et al*., 2009).

AMD is more likely to be found in Caucasians than in persons of African descent (Clemons *et al*., 2005). However, this conclusion was reached after studying African Americans within the United States, and only a few studies have started to investigate the prevalence of AMD in Africa (e.g. Ziskind *et al*., 2008; Nwosu, 2011; Oluleye, 2012). Importantly, studies on AMD-related FH polymorphisms in sub-Saharan African revealed a marked difference in geographical distribution suggesting that pathogen infections might represent a driving force for these regional differences (Ermini *et al*., 2012). In view of these findings, the implication of FH polymorphisms on the complement evasion efficiency of malaria parasites and potential links between the occurrence of FH polymorphisms and the incidences of malaria infections in malaria-endemic regions of Africa would be worth exploring.

We also showed that besides FH and FHL-1, the ABS parasites are able to bind CFHR-1. Such binding was not detected by the extracellular gametes of *P. falciparum*, probably because of the fact that CFHR-1 is quickly degraded in the mosquito midgut following the blood meal (Simon *et al*., 2013). The CFHR-group comprises five members, CFHR-1 to CFHR-5; each member of this group binds to C3b and can interact with the surfaces of a variety of pathogens [reviewed in Józsi *et al.* (2015)]. The precise role of each CFHR protein in complement activation and the exact contribution to disease pathology, however, is still unclear (Skerka *et al*., 2013). For example, CFHR-1, CFHR-2 and CFHR-5, which circulate in the blood as dimers or tetramers, do not exhibit co-factor activity and thus are unable to confer protection from complement activation. Further a recent study described that CFHR-3 acts as an antagonist of FH during complement evasion of the meningococcal pathogen *Neisseria meningitidis* and supports TCC formation (Caesar *et al*., 2014). It was further observed that the CFHR proteins preferentially bind to patterns of microbe surfaces (Blaum *et al*., 2015; reviewed in Józsi *et al*., 2015), an observation also made in this study, when we showed a prominent CFHR-1 binding to iRBCs, but not to RBCs. In a recent review, Józsi *et al.* (2015) thus postulated that the CFHR proteins might have evolved as decoys to reduce the amount of FH that can be bound by the pathogens, in consequence supporting their elimination by the human complement. Noteworthy, homozygous deficiency of CFHR-1 and CFHR-3 is very high (up to 16%) in African populations as compared with European populations (about 4%) (Holmes *et al*., 2013); again, the effect of CFHR-deficient sera on complement evasion by malaria parasites and potential links between CFHR deficiencies and incidences of malaria infections in malaria-endemic regions of Africa might be worth investigating.

It is now our aim to identify the receptor or the receptors involved in binding of FH and FH family proteins to the ABS surface. We currently expect that more than one receptor is involved in FH binding by the parasite, because both the free MZs as well as the schizont-iRBCs

are able to bind FH, and to date, no plasmodial protein is known that is present on both surfaces.

Surface-bound microbial complement receptors represent excellent vaccine targets, because antibodies binding to these receptors on the one hand impair complement evasion by the microbe and on the other hand activate the classical complement pathway. The use of microbial FH-receptors as vaccine candidates is currently been studied for meningococcal infections (Malito *et al.*, 2013; Rippa *et al.*, 2015), and crucial for such approach the vaccine candidate has to be mutated such that the protein loses its FH-binding function in order not to interfere with the human complement system.

In-depth knowledge on complement evasion by the ABSs and the identification of FH-binding receptors thus represents a first step towards measures to interfere with the erythrocytic replication cycle by promoting the human complement to eliminate the parasites. These pieces of evidence would contribute towards the development of a vaccine capable of inhibiting FH-mediated complement evasion of ABS and sexual stage parasites, in consequence preventing both progression and transmission of *falciparum* malaria.

Experimental procedures

Complement factors and antibodies

Purified human FH and factor I were purchased from Sigma-Aldrich. Polyclonal goat anti-human FH antibodies (directed against CCP1–20) and purified C3b were purchased from Calbiochem. Mouse mAb anti-human FH (isotype IgG2bκ; clone 131X; in PBS without preservatives; directed against CCP8-15), mouse mAb anti-C5b-C9 and polyclonal goat anti-human C3 antibody were purchased from Quidel. Polyclonal rabbit anti-human C4BP antibody was kindly provided by A. Blom (Lund University) (Happonen *et al.*, 2009). Mouse mAb anti-human HA (clone 12CA5; without preservatives or stabilizers) was purchased from Roche. Expression of recombinant peptides CCP1-4, CCP1-5, CCP1-6, CCP6-8, CCP15-19, CCP8-20 and CCP18-20 in *Pichia pastoris* was described elsewhere (Kühn *et al.*, 1995; Kühn and Zipfel, 1996). Generation of the polyclonal rabbit anti-CFHR-1 antisera was described elsewhere (Heinen *et al.*, 2009).

Parasite culture

P. falciparum ABSs of strains NF54 (gametocyte-producing) or F12 (gametocyte-deficient) were cultured in RPMI-HEPES culture medium supplemented with 50 μg ml^{-1} hypoxanthine (Sigma-Aldrich) and 10 μg ml^{-1} gentamicin (Gibco) at 5% hematocrit. The medium contained 0.5% Albumax II (Gibco) or 10–20 vol% human A+ serum (HIS, inactivated for 1 h at 55°C, or NHS) (Trager and Jensen, 1977; Ifediba and Vanderberg, 1981). All cultures were maintained at 37°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Human erythrocyte concentrate and serum were purchased from the Department of Transfusion Medicine, University Hospital Aachen, Germany. The erythrocyte and sera samples were pooled, and the donors remained anonymous; the work on human blood was approved by the ethics commission of RWTH Aachen University. To synchronize the ABSs, parasite cultures with 3–4% ring stages were centrifuged; the pellet was resuspended in five times pellet's volume of 5 vol% sorbitol (AppliChem) in ddH_2O and incubated for 10 min at room temperature (RT) (Lambros and Vanderberg, 1979). The cells were washed once with cell culture medium to remove the sorbitol, diluted to 5 vol% hematocrit with cell culture medium and further cultivated as described in the preceding texts. Schizont-iRBCs were purified using Percoll gradient centrifugation as described (Radfar *et al.*, 2009). To gain access to live MZ, schizont-iRBCs were either treated with 0.05% saponin in incomplete cell culture medium (without serum) for 5 min at 37°C or the MZ were purified using a published protocol (Boyle *et al.*, 2010). Here, schizont-iRBCs were incubated with incomplete medium containing 10 μM E64 (Sigma-Aldrich) for 8 h at 37°C. When the mature schizont stage was reached, the cells were centrifuged for 10 min at 1900× g and resuspended in 5 ml of incomplete medium via a syringe. The MZ were then filtrated through a 1.2-μm filter (VWR) and counted using a Neubauer chamber.

Replication and gametocytogenesis assays

To determine ABS replication, a synchronized ring stage culture of strain NF54 with a parasitemia of 0.5% was cultivated with cell culture medium containing 10 vol% NHS or HIS during a period of 0–120 h at 37°C. Medium was exchanged every 12 h to ensure the presence of active complement throughout the experiments. Simultaneously, samples were taken every 12 h and Giemsa smears were performed. The parasitemia was determined in 200 RBCs. To identify the blood stages present in the culture at a given time point, 100 iRBCs were counted per setting using a Zeiss LSM 510 at 100× magnification. To determine gametocytogenesis, a synchronized ring stage culture of strain NF54 with a parasitemia of 5% was cultivated in cell culture medium containing 10 vol% NHS or HIS at 37°C during a period of 0–240 h. Simultaneously, medium was exchanged and samples were taken every 24 h for Giemsa smear preparation. Gametocytemia was determined per 1000 RBCs, and the gametocyte stages II–V at the different time points were recorded. The experiments were performed in triplicate; data analysis was performed using MS Excel 2010 and GraphPad Prism 5.

Pig erythrocyte lysis assay

Cell culture medium with 20 vol% of freshly thawed NHS or of HIS was incubated for different time periods (30 min, 1, 6, 12, 16, 20 and 24 h) at 37°C. A total number of 5×10^6 pig erythrocytes was added to 100 μl of cell culture medium containing NHS or HIS and incubated for 1 h at 37°C. Subsequently, samples were centrifuged at 3400× g for 1 min at RT. The supernatants were collected and transferred to a 96-well plate. The amount of haemoglobin in the supernatant because of RBC lysis was

determined by measuring the absorbance at $OD_{405\,nm}$. Erythrocyte lysis was expressed as a percentage of maximum haemoglobin release as compared with lysis of pig RBCs by suspension in ddH_2O (set to 100%). The experiments were performed in triplicate; data analysis was performed using MS Excel 2010 and GraphPad Prism 5.

Indirect immunofluorescence assay

ABS cultures of strains NF54 and F12 containing intact or saponin-permeabilized schizont-iRBCs were incubated with cell culture medium containing 20 vol% NHS for 1 h at 37°C. After 3× washing with PBS, the cells were air-dried on Teflon slides and fixed in 4% paraformaldehyde/PBS or the cells were fixed in 4% paraformaldehyde/0.0025% glutaraldehyde/PBS in suspension for 10 min at RT. The fixed cells were subsequently blocked with 3% BSA/PBS for 1 h at RT and then incubated for 2 h at 37°C with polyclonal goat anti-CCP1-20 antibody, polyclonal rabbit anti-CFHR-1 or anti-C4BP antibody, mouse mAb anti-C5b-C9 or polyclonal goat anti-human C3 antibody in 0.5% BSA/PBS. For negative control, the fixed cells were incubated with NMS. Binding of primary antibody was visualized using Alexa Fluor 488-conjugated goat anti-mouse, chicken anti-goat or goat anti-rabbit secondary antibody (Molecular Probes) depending on the primary antibody used. The ABSs were routinely double-labelled with polyclonal rabbit anti-MSP1 antibody in combination with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody for 2 h at 37°C. If no double-labelling of the ABSs with anti-MSP1 antibody occurred, the RBCs were counterstained with 0.05% Evans Blue/PBS (Sigma-Aldrich) for 1 min at RT. Nuclei were stained with Hoechst 33342 according to the manufacturer's protocol (Invitrogen). Specimens were examined by Leica AF 6000 microscope. Digital images were processed using Adobe Photoshop CS software.

Western blot analysis

A total number of 2×10^7 per 100 μl schizont-iRBCs or purified MZ of strain F12 were incubated in cell culture medium containing 20 vol% NHS, NHS inactivated by 20 mM EDTA (Des Prez *et al*., 1975; Johnson *et al*., 2008) or HIS for 1 h at 37°C. For trypsin treatment, the cells were incubated in cell culture medium containing 100 $\mu g\,ml^{-1}$ trypsin for 30 min at 37°C, followed by 5× washing with incomplete medium, prior to incubation with 20 vol% NHS. Following 3× washing with PBS to eliminate unbound FH, the cell pellets were resuspended in 2× SDS sample buffer and heated for 10 min at 94°C. Under non-reducing conditions, the sample buffer lacked DTT and β-mercaptoethanol. Lysed niRBCs were used as a negative control. Following gel electrophoresis, separated parasite proteins were transferred to a nitrocellulose membrane according to the manufacturer's protocol (Carl Roth). Membranes were blocked to avoid unspecific binding by incubation with 5% skim milk powder/1% BSA/TBS, pH 7.5, followed by immunoblotting with the polyclonal goat anti-CCP1-20 antibody, polyclonal rabbit anti-CFHR-1 antibody, mouse mAb anti-C5b-C9, polyclonal goat anti-glycophorin A antibody (Sigma-Aldrich) or polyclonal goat anti-human C3 antibody for 2 h at RT. After washing, membranes were incubated with the respective alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) for 1 h at RT and developed in a solution of nitroblue tetrazolium chloride (NBT) and 5 brom-4-chlor-3-indoxylphosphate (BCIP; Sigma-Aldrich). Scanned blots were processed using Adobe Photoshop CS software, and band intensities were measured using the ImageJ programme version 1.44 p (NIH); data analysis was performed using MS Excel 2010.

ELISA for TCC detection

A total number of 5×10^7 schizonts-iRBCs of strain F12 was incubated with 100 μl of cell culture medium containing 20 vol% NHS or HIS for 1 h at 37°C and washed 3× with PBS. The cells were spun down; resuspended in sodium carbonate-coating buffer, pH 9.0; and transferred to a 96-well plate (ELISA high binding; Greiner). The wells were blocked by incubation with 3% skim milk powder/PBS over night at 4°C, followed by incubation with mAb anti-C5b-C9 for 2 h at 37°C. After 3× washing with PBS, the wells were incubated with an alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) for 1 h at 37°C. The reaction was developed with p-nitrophenylphosphate according to the manufacturer's protocol (Sigma-Aldrich), and the absorbance was measured at $OD_{450\,nm}$. The experiments were performed in triplicate; data analysis was performed using MS Excel 2010.

FH deletion mutants binding assay

A total number of 2×10^7 schizont-iRBCs of strain F12 was resuspended in 100 μl PBS containing 10 $ng\,\mu l^{-1}$ of the FH deletion mutants (CCP1-4, CCP1-5, CCP1-6, CCP6-8, CCP15-19, CCP18-20 and CCP8-20) or FH (CCP1-20) and then incubated for 1 h at 37°C. The cells were washed 3× with PBS; the pellet was then subjected to non-reducing gel electrophoresis followed by Western blot analysis using polyclonal goat anti-CCP1-20 antibody as described in the preceding texts.

Cofactor Assay

A total number of 2×10^7 schizont-iRBCs of strain F12, cultivated in 50 μl of Albumax-supplemented cell culture medium, were washed with incomplete cell culture medium and then incubated in the same volume of incomplete cell culture medium in the presence or absence of 125 ng purified FH/PBS for 30 min at 37°C. Schizonts were briefly washed with PBS, resuspended in 50 μl of incomplete cell culture medium and incubated with 250 ng purified C3b in the presence or absence of 30 ng purified factor I for another 30 min at 37°C. In the absence of complement factors, an equivalent volume of PBS was added. The cells were washed with PBS and subjected to gel electrophoresis followed by Western blot analysis using goat anti-human C3 antisera.

Growth inhibition assay

A synchronized ABS culture of strain F12 containing schizont-iRBCs with a parasitemia of 2.5% was cultivated in cell culture medium containing 20 vol% NHS or HIS or 10% Albumax as a

control in the presence or absence of 100 μg ml^{-1} mAbs anti-FH or anti-HA for 24 h 37°C. In the absence of antibody, an equivalent volume of PBS was added to the cultures. Medium (containing the mAbs or PBS) was exchanged after 12 h to ensure the presence of active complement throughout the experiments. Giemsa smears were performed to determine the parasitemia at 24 h post-seeding. The supernatant of the cultures was collected, and 100 μl were transferred to a 96-well plate. The amount of haemoglobin in the supernatant because of RBC lysis was determined by measuring the absorbance at $OD_{405\ nm}$. The experiments were performed in in triplicate; data analysis was performed using MS Excel 2010.

Gelatin flotation purification

Knob-infected RBCs were purified via gelatin purification as described (Goodyer *et al*., 1994). A total number of 5×10^7 schizont-iRBCs of strain F12 were resuspended in cell culture medium containing 0.7% gelatin and incubated for 1 h at 37°C. Subsequently, the supernatant was removed carefully without touching the pellet. The cells of the supernatant were spun down for 1 min and washed 3× with incomplete medium. The gelatin-purified schizont-iRBCs were counted using a Neubauer chamber. Data analysis was performed using MS Excel 2010 and GraphPad Prism 5.

Microwell adhesion assay

The CD36-binding behaviour of schizont-iRBCs was investigated as described (Prudhomme and Sherman, 1999). A 96-well plate (ELISA high binding; Greiner) was coated with 10 μg ml^{-1} of active recombinant CD36 (Thermo Fischer Scientific) overnight at 4°C. Following 3× washing with PBS, 1×10^7 schizont-iRBCs per well, resuspended in 100 μl of binding buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$,50 μg ml^{-1} BSA), were added and incubated for 2 h at 37°C. After 3× washing with PBS, the relative numbers of bound parasites was determined using the Malstat assay, which assesses the plasmodial lactate dehydrogenase activity (Goodyer and Taraschi, 1997). A volume of 100 μl of the Malstat reagent (1% L-lactate and 33% 3-acetylpyridine adenine dinucleotide in Tris–HCl, pH 9; Sigma-Aldrich) was added per well and mixed with 20 μl of a mixture of NBT (Nitro Blue Tetrazolium)/Diaphorase (1:1; 1 mg ml^{-1} stock each). The absorbance was measured at $OD_{630\ nm}$. The experiments were performed in in triplicate; data analysis was performed using MS Excel 2010 and GraphPad Prism 5.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1: Effect of human complement on ABS differentiation.

Fig. S2: Pig RBC lytic activity of NHS.

Fig. S3: Effect of human complement on gametocyte differentiation.

Fig. S4: IFAs of NMS and saponin-permeabilization controls.

Fig. S5: Binsding of C4BP to the surface of pathogens.

Fig. S6: Determination of purity of FH deletion mutant peptides.

Fig. S7: Evaluation of knob formation in P. falciparum strain F12.

Fig. S8: Binding of FH by ABS parasites following trypsin-treatment.