Complement and Antibody-mediated Enhancement of Red Blood Cell Invasion and Growth of Malaria Parasites

Sergei Biryukov, Evelina Angov, Mary E. Landmesser, Michele D. Spring, Christian F. Ockenhous, and José A. Stoute

1. Introduction

Malaria, a mosquito-borne infectious disease caused by eukaryotic intracellular protists of the genus *Plasmodium*, kills close to one million people worldwide each year, predominantly children under 5 years of age (Murray et al., 2012; World Health Organization, 2013). Of the five species of *Plasmodium* that can infect humans, infection with *Plasmodium falciparum* accounts for the vast majority of deaths worldwide. *Plasmodium*’s complex life cycle involves invasion of hepatocytes and red blood cells (RBCs); however, the clinical symptoms arise from the invasion of RBCs by the asexual blood stage parasite. Antibodies are thought to play an important role in natural immunity as demonstrated by the reduction in parasitemia and clinical symptoms in *P. falciparum*-infected individuals following passive transfer of immunoglobulins from semi-immune donors (Cohen et al., 1961; McGregor, 1964a; Sabchareon et al., 1991). However, the effector mechanisms are poorly understood.

Development of a vaccine to block RBC invasion has proven to be an elusive goal. Much of the effort has been focused on the merozoite surface protein 1 (MSP1) and the apical membrane antigen 1 (AMA1). Multiple preclinical vaccine studies have demonstrated, using growth inhibition assays (GIA), that antibodies targeting MSP1 and AMA1 of *P. falciparum* have in vitro RBC invasion and growth inhibitory activity (Angov et al., 2003; Chang et al., 1992; Kennedy et al., 2002). In addition, some degree of protective immunity has been seen in some animal models (Darko et al., 2005; Singh et al., 2003; Singh et al., 2006). Unfortunately, to date, these studies have not translated into in vivo efficacy in human vaccine trials (Ogutu et al., 2009; Sagara et al., 2009; Spring et al., 2009). Thus, GIA is a poor predictor of blood stage protective immune responses despite the fact that antibodies do inhibit RBC invasion. The reasons for this discrepancy are unknown.

One possible explanation for this discrepancy came to light as the result of the discovery that the complement receptor 1 (CR1) is a sialic acid (SA)-independent receptor for *P. falciparum* (Spadafora et al., 2010; Tham et al., 2010). The complement system is part of the innate immune response and is an important effector arm of humoral immunity. It can be activated via three main pathways: the classical pathway (CP); the lectin pathway (LP); and the alternative pathway (AP).
(Ricklin et al., 2010). Once activated, the complement system induces the formation of opsonins (C3b, C4b) that promote phagocytosis, induce lysis by formation of the terminal complement complex (TCC), and promote an inflammatory response (Ricklin et al., 2010). Once bound to the pathogen, surface C3b and C4b serve as ligands for CR1, which is present on RBCs as well as most leukocytes (Fearon, 1980; Tas et al., 1999). CR1 also binds complement factors C1q and mannan-binding lectin (MBL) (Ghiran et al., 2000; Tas et al., 1999).

We hypothesize that *P. falciparum* is capable of exploiting the opsonizing qualities of complement deposition on the merozoite surface which will allow it to bind to CR1 and invade via this invasion pathway. If we are correct, complement activation could negate the inhibitory activity of anti-merozoite neutralizing antibodies generated post vaccination or during natural infection.

2. Materials and Methods

2.1. Parasites, Parasite Culture, and RBC Treatment

SA-independent strains (7G8, 3D7, HB3, and Dd2NM) were obtained from the Walter Reed Army Institute of Research. SA-dependent strains (FVO, Camp, Dd2, and FCR3) were obtained from the Malaria Research Reference Reagent Resource Center (BEI Resources, Manassas, VA). Parasite cultures were maintained at 1–4% hematocrit (Hct) in O+ blood with 10% heat-inactivated (HI) plasma in RPMI 1640 Medium and Reference Reagent Resource Center (BEI Resources, Manassas, VA). Parasite cultures were maintained at 1–4% hematocrit (Hct) in RPMI 1640 Medium (Sigma-Aldrich, St. Louis, MO) with 25 μg/ml gentamicin, 20 μg/ml hypoxanthine, and 7.5% w/v NaHCO3 (complete media) in malaria gas (Ghiran et al., 2000; Tas et al., 1999). SA-dependent strains (7G8, 3D7, HB3, and Dd2NM) were obtained from the Walter Reed Army Institute of Research. SA-dependent strains (FVO, Camp, Dd2, and FCR3) were obtained from the Malaria Research Reference Reagent Resource Center (BEI Resources, Manassas, VA). Parasite cultures were maintained at 1–4% hematocrit (Hct) in O+ blood with 10% heat-inactivated (HI) plasma in RPMI 1640 Medium (Sigma-Aldrich, St. Louis, MO) with 25 μg/ml gentamicin, 20 μg/ml hypoxanthine, and 7.5% w/v NaHCO3 (complete media) in malaria gas (5% O2, 5% CO2, and 95% N) at 37 °C. Cultures were synchronized twice a week by 5% sorbitol lysis (Lambros and Vanderberg, 1979). Neuraminidase treatment of RBCs was carried out as described (Spadafora et al., 2010).

2.2. Sera, Complement Factors, and CH50/AS50 Assay

Non-hemolyzed whole blood was collected with a 21 gauge needle from two O+ volunteers into glass tubes without additives (Recton Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature for 50 min. The sera were centrifuged at 1300 × g for 15 min and the serum was removed and re-centrifuged for 5 min again to pellet any residual RBCs or clot particles. The serum was aliquoted and stored at −80 °C. Serum was used fresh (FS) or after heat inactivation (HS) at 56 °C for 30 min in 200 μl aliquots in 1.5 ml polypropylene microcentrifuge tubes (Denville Scientific, South Plainfield, NJ). Serum C2 and Factor B (FB) were selectively inactivated by incubation of 200 μl serum aliquots in 1.5 ml microcentrifuge tubes in a 56 °C water bath for 3 min with constant mixing (Araujo et al., 1991). Purified complement factors (C1q, C2, C3, C4) and selectively depleted or inactivated sera were obtained commercially (CompTech, Tyler, TX), 50% CP and AP complement hemolytic (CH50 and AH50) assays were performed using the standard methods described in the literature (Morgan, 2000).

2.3. In Vitro Invasion Assays

Invasion assays were carried out in triplicate wells of a 96-well plate containing sorbitol-synchronized cultures of late trophozoites or schizonts at 0.5% to 2% parasitemia in 2% sorbitol-synchronized cultures of late trophozoites or schizonts (Spadafora et al., 2010). Inhouse prepared serum (FS or HIS) was always commercially-obtained (CompTech, Bristol, UK) (Mastellos et al., 2015), was used to determine the effect of blocking C3. A peptide derived from the linearized and scrambled sequence of compstatin without cysteines (NH3-Arg-Thr-Ala-Trp-Cln-His-Asp-Ala-Ile-His-Val-Gly-Val-COOH) was synthesized and used as a control. CR1 was used as an inhibitor (Cellidex Therapeutics, Hampton, NH) and fetuin (Sigma-Aldrich, St. Louis, MO), an inert glycoprotein with no complement activity, was used as negative control protein where appropriate. Antibodies were added at different concentrations. Mouse monoclonal antibody mAb5.2 was raised against the 19 kDa subunit of the merozoite surface protein 1 (MSP119) (Siddiqui et al., 1986) and was purified from cultured hybridoma by protein A/G chromatography (Thermo Fisher Scientific, Rockford, IL). Mouse IgG2b Clone eB2mg2b (ebioscience, San Diego, CA) was used as isotype control. IgG from individuals vaccinated with MSP142 (Otsyula et al., 2013), comprising the C-terminal 42-kDa portion of the FVO variant of *P. falciparum* MSP1, was purified by protein A/G chromatography. The plate was placed in a gas-impermeable heat-sealable bag and inflated with malaria culture gas (Haynes et al., 2002). As an alternative procedure we used filter-purified merozoites as described by Boyle et al. (2010, 2015) with the exception that late trophozoite/schizonts were enriched using a Percoll gradient (Moll et al., 2008). After 20 to 24 h, 5 μl aliquots of individual wells were added to 100 μl 5 μg/ml Hoechst 33342 (Life Technologies, Grand Island, NY) in PBS containing 2% paraformaldehyde (Sigma-Aldrich). At least 100,000 RBCs were acquired for each sample. Acquisition was done using a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a violet laser and analysis was performed using FCS Express (De novo Software, Glendale, CA). RBCs with Hoechst-positive ring stage parasitemia (early trophozoite) were used as endpoint for 24-h invasion assays. The background staining of an unfixed RBC sample was subtracted. The majority of the experiments were repeated 2–3 times.

2.4. Merozoite Attachment Assay

Unless otherwise stated, all the centrifugation steps were at 770 × g for 5 min at room temperature (RT). Attachment assays were carried out as invasion assays except that highly synchronous late stage parasite cultures were incubated with 10 μg/ml of leupeptin (Sigma-Aldrich) for 6 to 8 h followed by three washes with RPMI 1640. The hematocrit was adjusted to 2% by the addition of fresh RBCs. The final culture medium also contained 2 μM cytochalasin D (Sigma-Aldrich) and 10% complement deficient or reconstituted serum in the presence or absence of mouse mAb5.2 or IgG2b. The cultures were incubated again for 3–4 h at 37 °C in malaria gas followed by centrifugation at 400 × g for 1 min at 4 °C and two washes with 100 μl of 1% BSA/PBS blocking buffer, mAb5.2 (2 mg/ml), if not added previously, and goat polyclonal anti-C3 (MP Biomedicals, Santa Ana, CA) antibodies diluted 1:33 in blocking buffer were added for 30 min at 4 °C. Following three washes with blocking buffer, the pellets were resuspended in 1:100 dilution of donkey anti-goat IgG-PerCP R&D Systems, Minneapolis, MN) and goat anti-mouse DyLight 488 (KPL, Gaithersburg, MD) in blocking buffer and incubated for 30 min at 4 °C. After an additional three washes with blocking buffer the pellets were resuspended in 2% paraformaldehyde/PBS with 5 μg/ml Hoechst 33342 (Life Technologies) solution. Acquisition by flow cytometry was carried out as above.

2.5. Confocal Microscopy of Merozoite Attachment

For confocal microscopy of the interaction of merozoites with RBCs the culture conditions were the same as for attachment assays. After incubation, the pellets were resuspended in 1% BSA/PBS blocking buffer containing 130 μg/ml mouse anti-MSP1 mAb5.2 (if not present during the assay), 190 μg/ml of chicken polyclonal anti-CR1 (Gallus Immunotech, ON, Canada), and 30 μg/ml of mouse monoclonal IgH anti-C3 IgG1 (Kerafast, Boston, MA) and incubated for 30 min at 4 °C. The specificity of each primary antibody was verified by the use of negative control antibodies. After three washes with blocking buffer, the pellets were resuspended in 100 μl of blocking buffer containing 1:500...
dilutions of goat anti-mouse IgG2b-DyLight 488, goat anti-chicken Alexa Fluor 546, and goat anti-mouse IgG1 Alexa Fluor 594 (Thermo Fisher Scientific) in the presence of 30 μM Vybrant DID cell labeling solution (Life Technologies) followed by an incubation for 30 min at 4 °C. After an additional three washes, the pellets were resuspended in 4% paraformaldehyde with 10 μg/ml Hoechst 33342 (Life Technologies). Prior to imaging, 10 μl of the cell suspension was transferred to 0.6 ml microcentrifuge tubes and centrifuged at 200 × g for 1 min and the pellet was resuspended in 10 μl of VectorShield Hard Set (Vector Labs, Inc., Burlingame, CA). 4 μl of this suspension was mounted on a slide, covered with a cover slip, and allowed to harden for a few minutes. Fluorescence z-series were collected using a LEICA SP8 confocal microscope (Leica Microsystems Systems Inc., Buffalo Grove, IL) at 40× oil (HC PL APO CS2 40×/1.30 Oil) immersion. The images were processed using Imaris (Bitplane, Concord, MA) image processing software.

2.6. Passive Transfer Experiments

Polyclonal anti-Plasmodium berghei antibody was generated by three cycles of infection and treatment of C57BL/6 J mice (The Jackson Laboratory, Bar Harbor, ME). For the initial infection mice were injected intraperitoneally with 1 × 10⁶ P. berghei ANKA IRBC. Parasitemia was monitored with Giemsa smears and once it reached (5%–10%) the mice were treated with sub-curative one intramuscular dose of chloroquine (500 μg/mouse) for three consecutive days. The parasitemia was allowed to rebound and the cycle was repeated for a total of 3 times. For the final treatment, mice were treated daily for one week and allowed to rest for two weeks. Plasma was collected from mice by cardiac puncture in citrate phosphate dextrose solution (CPD) (Sigma-Aldrich) and total antibody was purified using protein A/G columns (GenScript, Piscataway, NJ). Antibody purified from uninfected mice served as control. For passive transfer experiments wild type or C3 deficient mice of C57BL/6J background were injected with 1.5 × 10⁷ IRBCs in 100 μl of RPMI 1640 in one retro-orbital plexus and with 100 μl of anti-Pb, control antibodies, or 100 μl of PBS in the contralateral plexus. Parasitemia was monitored by staining tail vein blood with Hoechst 33342 (2 μg/ml in PF) (Life Technologies) at pre-determined intervals until day 7 post-infection. Acquisition and analysis were performed as described above.

2.7. Ethics Statement

The use of human serum and plasma reported here was carried under human subject research exemptions by the Walter Reed Army Institute of Research Human Subject Research Review Board and by the Penn State Hershey Medical Center Institutional Review Board that allowed the use of unidentified serum and plasma samples. The malaria vaccine serum samples were collected originally using protocols approved by the Walter Reed Army Institute of Research Human Subject Review Board and adhered to the principles of the Declaration of Helsinki. All animal experiments were carried under protocols approved by the Penn State Hershey Medical Center Institutional Animal Care and Use Committee and adhered to NIH guidelines for care and use of laboratory animals.

Fig. 1. FS and anti-merozoite monoclonal antibody mAb5.2 enhance P. falciparum invasion of RBCs relative to HIS. a) Two SA-independent strains of P. falciparum, 7G8 and 3D7, were tested in the presence of 10% FS, 3 min HIS, 5 min HIS, or 30 min HIS. Invasion of RBCs decreased with progressively longer heat inactivation times. b) Invasion of RBCs in the same experiment as panel A expressed as percent enhancement relative to 30 min HIS. See Fig. S3 for additional replicates. c) Invasion of P. falciparum strain 7G8 was tested in the presence of monoclonal anti-merozoite surface protein 1 (MSP1) mAb5.2 (40 μg/ml), an isotype control antibody (IgG2b), or PBS in 10% FS or 30 min HIS. Invasion of RBCs is expressed as percent enhancement in FS relative to 30 min HIS. Each panel depicts a representative experiment. See Fig. S5 for absolute parasitemia and one additional replicate. Error bars represent standard deviations for triplicate wells. P values were obtained by one-way ANOVA with post-hoc comparisons.
2.8. Statistical Analysis

Statistical analysis was done with SigmaPlot v11.2 (Systat Software, San José, CA). Means of normally distributed continuous numeric variables from two groups were compared using t-test or paired t-test, whichever was appropriate. Comparisons across more than two groups were done using analysis of variance with post-hoc Holm-Sidak tests. Differences in categorical variables were analyzed using chi-square with Fisher’s exact test. All tests were two-tailed \( \alpha < 0.05 \).

3. Results

3.1. Invasion of RBCs is Enhanced by Fresh Serum and Anti-merozoite Monoclonal Antibody mAb5.2 Relative to Heat-inactivated Serum

To test the hypothesis that complement can enhance RBC invasion, we first evaluated the effect of heat inactivation of serum on invasion. Although traditional methods of complement inactivation utilize 30 min heat treatment at 56 °C, this duration of heat treatment likely results in the inactivation of multiple enzymes in the complement pathway. Thus, in order to achieve a more selective inactivation, we also incubated serum for 3 min and 5 min at 56 °C (Araujo et al., 1991) and confirmed inactivation by measuring CH50 and AH50 (Fig. S1A and S1B). Fig. 1a shows the ring parasitemia of overnight RBC invasion in the presence of 3, 5 or 30 min HIS or FS. Since heat inactivation of serum is standard in most malaria cultures or GIA (Moll et al., 2008), from this point forward we present the data as enhancement relative to 30 min HIS (Fig. 1b). The use of FS resulted in a 40% invasion enhancement relative to 30 min HIS. This enhancement was not due to excessive RBC lysis in the FS wells since the level of free hemoglobin was very low and similar between HIS and FS (Fig. S2). A drastic reduction in invasion enhancement was observed with just 3 or 5 min HIS (Fig. 1b). Additional replicates are shown in Fig. S3. These results suggest that complement may be involved in mediating the enhancement of invasion observed in FS relative to HIS. Further, we were also able to identify enhanced C3 deposition on single merozoites after incubation in FS but not in 30 min HIS (Fig. S4).

In order to determine whether activation of complement via the classical pathway by anti-merozoite antibodies could enhance invasion, we used mAb5.2, raised against the C-terminal 19 kD fragment of \textit{P. falciparum} Uganda-Palo Alto MSP1 (Kaslow et al., 1994; Siddiqui et al., 1986). This antibody was selected due to its availability and relative ease of production. Fig. 1c shows that addition of mAb5.2 resulted in significant enhancement of invasion above fresh serum. Fig. S5 shows the absolute parasitemia for Fig. 1c and one additional replicate.

3.2. Enhancement of Invasion by Anti-merozoite Monoclonal Antibody mAb5.2 is Complement-dependent

Using CH50 we confirmed that we could reverse the 3 min heat inactivation of complement by addition of C2 and \( \alpha \)B rescued RBC invasion enhancement properties of mAb5.2 in 3 min HIS. Invasion of \textit{P. falciparum} strain 7G8 was tested in the presence of 40 \( \mu \)g/ml anti-MSP1 mAb5.2, 40 \( \mu \)g/ml IgG2b isotype control, or PBS in the presence of 10% FS, 3 min HIS, or 30 min HIS. C2 and \( \alpha \)B were added to a final concentration of 25 \( \mu \)g/ml and 200 \( \mu \)g/ml respectively. The glycoprotein fetuin was used as a control at 225 \( \mu \)g/ml. Fig. S6A shows the absolute parasitemia. Additional replicates are shown in Fig. S7. b) Enhancement of RBC invasion by anti-MSP1 mAb5.2 was eliminated by the peptide complement inhibitor compstatin but not by control peptide. Conditions were the same as panel A. Invasion of RBCs is expressed as percent enhancement relative to 30 min HIS. Fig. S9A shows the absolute parasitemia. Additional replicates are shown in Fig. S9B and S9C. Error bars represent standard deviations of triplicate wells. P is based on two-sample t-test for the comparison between mAb5.2 and IgG2b or PBS.

![Fig. 2. Enhancement of RBC invasion by anti-merozoite monoclonal antibody mAb5.2 is complement dependent. a) Addition of C2 and \( \alpha \)B rescued RBC invasion enhancement properties of mAb5.2 in 3 min HIS. Invasion of \textit{P. falciparum} strain 7G8 was tested in the presence of 40 \( \mu \)g/ml anti-MSP1 mAb5.2, 40 \( \mu \)g/ml IgG2b isotype control, or PBS in the presence of 10% FS, 3 min HIS, or 30 min HIS. C2 and \( \alpha \)B were added to a final concentration of 25 \( \mu \)g/ml and 200 \( \mu \)g/ml respectively. The glycoprotein fetuin was used as a control at 225 \( \mu \)g/ml. Fig. S6A shows the absolute parasitemia. Additional replicates are shown in Fig. S7. b) Enhancement of RBC invasion by anti-MSP1 mAb5.2 was eliminated by the peptide complement inhibitor compstatin but not by control peptide. Conditions were the same as panel A. Invasion of RBCs is expressed as percent enhancement relative to 30 min HIS. Fig. S9A shows the absolute parasitemia. Additional replicates are shown in Fig. S9B and S9C. Error bars represent standard deviations of triplicate wells. P is based on two-sample t-test for the comparison between mAb5.2 and IgG2b or PBS.](image)

![Fig. 3. Enhancement of RBC invasion by mAb5.2 is abolished in the presence of sCR1. Invasion of \textit{P. falciparum} strain 7G8 was tested in the presence of 40 \( \mu \)g/ml anti-MSP1 mAb5.2 or 40 \( \mu \)g/ml IgG2b isotype control in the presence of 10% FS or 30 min HIS. The final concentration of sCR1 or fetuin was 80 \( \mu \)g/ml. Fig. S10 shows the absolute parasitemia and additional replicates. Error bars represent standard deviations of triplicate wells. P is based on two-sample t-test.](image)
To further confirm that complement is responsible for antibody-mediated enhancement of RBC invasion, we used the C3-selective inhibitor compstatin, a cyclic 13-amino acid peptide that inhibits C3 cleavage and activation in human serum (Sahu et al., 1996). Enhancement of invasion was observed in FS in the presence of mAb5.2. The addition of compstatin eliminated this enhancement (Figs. 2b and S9). Interestingly, compstatin had no effect on the enhancement of invasion by FS alone. These results demonstrate that activation of the CP can result in enhancement of RBC invasion by merozoites.

3.3. Soluble CR1 Blocks Enhancement of Invasion by Fresh Serum and Anti-merozoite Antibodies

To test whether CR1 is involved in complement and antibody-mediated enhancement of RBC invasion by *P. falciparum* we used soluble CR1 (sCR1) as an inhibitor. Anti-merozoite antibody-mediated enhancement of RBC invasion was not onlynegated in the presence of sCR1 but declined below the level of the isotype control IgG2b (Figs. 3 and S10). There was no effect in the absence of mAb5.2 (Figs. 3 and S10).

3.4. SA-dependent Strains Demonstrate Greater Enhancement of Invasion in the Presence of Complement than SA-independent Strains

*P. falciparum* can be subdivided into SA-dependent strains that invade neuraminidase (NA)-treated RBCs at a low level and SA-independent strains that are able to invade NA-treated RBCs at a higher level (Mitchell et al., 1986) via CR1 and the merozoite ligand PfRh4 (Thamm et al., 2010). Since SA-dependent strains are unable to utilize PfRh4 for CR1-mediated invasion (Stubbs et al., 2005), we hypothesized that these strains may be more reliant on complement to accomplish this function. Hence, we compared invasion levels of SA-dependent and SA-independent strains in fresh and 30 min HIS in untreated and NA-treated RBCs. SA-dependent strains demonstrated greater enhancement of invasion with fresh serum compared to HIS in NA-treated RBCs than SA-independent strains, median enhancement 54.8% vs. 31.5%, *P* < 0.01 by two-sample *t*-test (Figs. 4a, S11, and S12). Similar results, although of lower magnitude, were observed in the presence of C3/C4-inactivated vs C3/C4-reconstituted heterologous pooled serum (Fig. 4b and S11C).

3.5. Anti-merozoite mAb5.2 Enhances Merozoite Attachment to RBCs and Induces CR1 Aggregation in a Complement-dependent Manner

To address the question of whether the presence of complement enhances the binding of merozoites to the RBC surface we performed attachment assays. In order to inhibit merozoite invagination we allowed merozoites to egress in the presence of cytochalasin D (Miller et al., 1979), complement deficient or reconstituted serum, and either mAb5.2 or IgG2b isotype control. We then detected RBCs with surface merozoites with mAb5.2 by flow cytometry. As expected, in the presence of mAb5.2 and deficient serum reconstituted with either C3/C4 or C3, we observed a significantly greater percent of RBCs with attached merozoites and surface C3 than in the presence of IgG2b isotype control and complement deficient serum (Fig. 5a and S13), *P* < 0.01 by two-sample *t*-test.

In the interest of depicting the direct interaction of merozoites with RBCs in the presence of complement and mAb5.2 we performed immunofluorescence microscopy. Attachment assays were setup in the presence of C3-depleted and reconstituted heterologous serum, with or without mAb5.2. In the presence of C3 and mAb5.2 there was dense aggregation of CR1 and lipids, and C3 deposition at the site of merozoite contact with the RBC but these findings were absent in C3-depleted serum, *P* < 0.001 by chi-square test (Fig. 5c, d, and e).
3.6. Complement Decreases Invasion Inhibitory Activity of Human Anti-MSP1 Antibodies

To assess the role of human antibody-mediated complement activation on RBC invasion we purified total IgG from MSP142 vaccines (Otsyula et al., 2013) and tested it in invasion assays using C3/C4-inactivated or reconstituted sera. Percent inhibition was calculated relative to PBS-only since we did not have pre-immunization serum. The median inhibitory activity in C3/C4-inactivated serum was negligible but upon reconstitution of serum with the missing complement factors the inhibitory activity became negative, signifying invasion enhancement (Fig. 6). There was no difference between C3/C4-inactivated and reconstituted serum in the presence of IgG from unvaccinated controls.

3.7. Passive Transfer of Anti-P. berghei Antibodies Enhances Parasitemia and C3 Deficiency Decreases Parasitemia in Mice

To validate the observed in vitro antibody-mediated complement enhancement of RBC invasion in an in vivo setting we infected C57BL/6 mice with P. berghei ANKA and administered varying concentrations of polyclonal total anti-P. berghei IgG (anti-Pb), control total IgG from naïve mice, or PBS. The anti-Pb antibody resulted in enhancement of endpoint parasitemia in a reverse dose-dependent manner when compared to the PBS control group (Fig. 7A and B). Additional replicates are found in Fig. S14. Surprisingly, control antibodies, independent of the dose administered, produced an inhibitory effect when compared to the PBS control, P = 0.06 by two-sample t-test.
To further explore the role of C3 in in vivo parasite growth, we subjected C3−/− mice to P. berghei infection in the presence of anti-Pb, control antibodies, or PBS. Fig. 7A and 7D show that C3−/− mice had blunted parasite growth relative to their wild type counterparts across all groups although it was only statistically significant in the case of PBS. C3−/− mice that received anti-Pb antibody still showed enhancement of parasitemia although it did not reach statistical significance. C3−/− mice that received control IgG had an intermediate response.

4. Discussion

Antibodies that coat pathogens in the circulation induce activation of the complement cascade and opsonization with C4b and C3b. Opsonized pathogens can bind to CR1 on RBCs via C4b and C3b. When the RBCs traverse through the liver or spleen and encounter macrophages, attached pathogens are removed by phagocytosis and the RBCs are spared and recirculate (Davies et al., 1990). This process of binding to RBCs and removal of opsonized pathogens is beneficial to the host. However, unlike other pathogens, opsonization of malaria merozoites may facilitate binding to their ultimate target cell, the RBC. Recently, two groups (Kennedy et al., 2016; Rosa et al., 2016) reported that the fluid phase complement regulator Factor H can bind to IRBCs and to merozoites. Factor H functions to accelerate the degradation of the AP convertase and as cofactor for the Factor I-mediated cleavage of C3b to iC3b (Pouw et al., 2015). Since CR1 is also able to bind iC3b (Nilsson et al., 2011), Factor H activity could lead to the accumulation of this molecule on the merozoite surface and enhance binding to CR1. Therefore, we reasoned that opsonization of merozoites may enhance RBC...
invasion. Enhancement of invasion by complement has been reported in other pathogens such as *Leishmania* (Da Silva et al., 1989; Datta and Rappaport, 2006; Schlesinger et al., 1990; Schlesinger, 1993).

To investigate the role of complement in RBC invasion by merozoites, we evaluated the effect of serum in the absence or presence of anti-merozoite antibodies. Because heat treatment at 56 °C is the traditional way to inactivate complement, we first compared FS to 56 °C HIS. We showed that FS enhances RBC invasion relative to 3, 5, and 30 min HIS. Although we were able to restore the CH50 activity of 3 min HIS by addition of C2 (Fig. S1A), addition of C2 and/or Fb to 3 min HIS did not rescue RBC invasion enhancing activity. One possible explanation is that 3 min heat inactivation may also inactivate elements of the LP upstream of C2 that are activated by merozoites such as mannose binding lectin associated serine proteases (MASPs).

In order to test the effect of anti-merozoite antibodies in complement-mediated RBC invasion we used two types of antibodies, an anti-merozoite mouse monoclonal (mAb5.2) and polyclonal IgG from MSP142 vaccines (Otsyula et al., 2013). Use of mAb5.2 resulted in enhancement of RBC invasion above that of FS alone. 3 min HIS eliminated this enhancement and addition of both C2 and Fb was required for rescue. Because C2 is an integral part of the CP and Fb of the AP amplification loop, these data provide strong evidence that both of these pathways of complement activation play a role in antibody-mediated enhancement of RBC invasion. The involvement of the CP is not surprising given its reliance on antibodies. However, antibodies are also known to play an important role in stabilizing the nascent AP convertase (Lutz and Jelezarova, 2006). In addition, the AP serves as the main amplification loop for the majority of complement deposition even when the CP is the initial source of complement activation (Harboe et al., 2004; Harboe and Molines, 2008). The important role of C3 was further demonstrated by the effect of the C3 specific inhibitor compstatin. Use of this inhibitor eliminated antibody-mediated enhancement of invasion (Fig. 2b). Although compstatin had no effect on enhancement by FS alone, we cannot rule out that other complement factors such as C1q, MBL, or C4 also play a role here. Our experiments using single factor depleted and reconstituted heterologous serum (Fig. S8) suggest that heterologous serum is not as effective as autologous FS in producing enhancement of invasion. Possible reasons include the presence of minor incompatibilities between serum and RBCs or residual effects from the method of depletion. Thus, further studies should be done with single factor-depleted autologous serum. Enhancement of invasion has been described with the use of antibodies that block other antibodies that inhibit the processing of MSP1 (Guevara Patino et al., 1997). However, the mechanism that we describe here is clearly different.

In an effort to validate the relevance of antibody and complement-mediated enhancement of RBC invasion in humans we purified total IgG from serum from MSP142 vaccine recipients collected two weeks after three doses of vaccine (Otsyula et al., 2013). Due to the limited amounts of sample obtained we were unable to carry out extensive assays. Nonetheless, we measured the RBC invasion inhibitory activity of total IgG in C3/C4-inactivated or reconstituted serum. Consistent with previous results (Otsyula et al., 2013), we found very little inhibitory activity in total IgG. However, unlike IgG from non-immunized controls, IgG from vaccine recipients showed reversal of inhibitory activity and enhancement of RBC invasion upon reconstitution of serum with C3/C4. Thus, these results demonstrate that enhancement of RBC invasion can occur with human anti-merozoite antibodies.

Contrary to our data, Boyle et al. (Boyle et al., 2015) recently reported that complement activation enhances the inhibitory activity of human anti-merozoite antibodies. They determined that the critical inhibitory step was fixation of C1q on the merozoite surface. One major difference between the approach of these investigators and ours is their use of filter-purified merozoites as opposed to allowing merozoites to naturally egress from schizonts. Consequently, we carried out invasion assays with filter-purified merozoites as described by Boyle et al. (Boyle et al., 2015). We showed that filtered-purified merozoites appear to be highly defective and more sensitive to complement activation than naturally egressed merozoites (Fig. S15).

To demonstrate the role of CR1 in the process of complement-mediated invasion we used sCR1 as a competitor. We observed that in the presence of sCR1 there was not only complete reversal of antibody-mediated enhancement in FS but significant amount of inhibition was observed in comparison to the isotype (Fig. 3). The inhibitory activity of sCR1 may be attributed to its diverse functions. Firstly, sCR1 competes for binding sites with erythrocyte surface CR1, which may potentially form large C3b-sCR1 complexes on the merozoite surface and induce steric hindrance of ligand receptor interactions in the presence of these complexes. Secondly, sCR1 is able to bind to PRRh4 on the merozoite surface thereby inhibiting the direct interaction of erythrocyte surface CR1 with PRRh4. Thirdly, sCR1 possesses complement regulatory activity by destabilizing membrane assembled C3 convertases through its intrinsic decay accelerating activity and by acting as a cofactor for Factor I, which breaks down C3b into iC3b. The role of CR1 in complement-dependent invasion was further supported by our co-localization studies that clearly show aggregation of CR1 at the site of merozoite attachment but only in the presence of C3.

PRRh4 has been proposed as the native merozoite ligand that binds to CR1. SA-dependent strains of *P. falciparum* are unable to use this ligand (Stubbs et al., 2005). Therefore, we hypothesized that SA-dependent strains may be more reliant on complement to use this invasion pathway. Although the invasion of NA-treated RBCs by SA-dependent strains is low, the percent enhancement of invasion in the presence of FS is significantly greater than SA-independent strains. These data demonstrate that, for some strains, C3 can act as the sole CR1 ligand.

Lastly, our passive transfer experiments in mice confirm that anti-malaria antibody and complement can benefit parasite growth. Administration of anti-Pb IgG enhanced parasite growth in a reverse dose-dependent manner. At first look, these results stand in contrast to previous studies that have shown that antibody from immune rodents inhibits growth in vivo when given to naïve animals (Cavinato et al., 2001; Jarra et al., 1986; Rotman et al., 1998; Waki et al., 1995; Yoneto et al., 2001). However, the doses of antibody in those studies were much higher, usually given in milligram amounts and sometimes in several doses prior to and after infection. While mice do not express CR1 on their RBCs, they do express other complement receptors that can bind complement-dependent enhancement in FS but signiﬁcant amount of inhibition was observed in comparison to the isotype (Fig. 3). The inhibitory activity of sCR1 may be attributed to its diverse functions. Firstly, sCR1 competes for binding sites with erythrocyte surface CR1, which may potentially form large C3b-sCR1 complexes on the merozoite surface and induce steric hindrance of ligand receptor interactions in the presence of these complexes. Secondly, sCR1 is able to bind to PRRh4 on the merozoite surface thereby inhibiting the direct interaction of erythrocyte surface CR1 with PRRh4. Thirdly, sCR1 possesses complement regulatory activity by destabilizing membrane assembled C3 convertases through its intrinsic decay accelerating activity and by acting as a cofactor for Factor I, which breaks down C3b into iC3b. The role of CR1 in complement-dependent invasion was further supported by our co-localization studies that clearly show aggregation of CR1 at the site of merozoite attachment but only in the presence of C3.

C3−/− mice consistently showed significant blunting of parasitemia relative to wild type mice. Yet, they still showed enhancement of parasite growth in response to anti-Pb IgG, probably due to residual C4b opsonization activity. Previous studies of the role of complement in the growth of rodent malaria have been inconsistent. Use of cobra venom factor (CVF) as a complement depleting agent resulted in increased parasitemia (Ward et al., 1981). However, CVF exerts its action by inducing AP convertase formation and C3 activation which could result in parasite growth enhancement by increased deposition of C3 fragments on merozoites. Unlike our study, a previous study (Ramos et al., 2012) reported no change in *P. berghei* parasitemia in C3−/− mice compared to wild type mice, but in that study quantitation was done by microscopy and infection was by intraperitoneal infection. C1q and C2/Fb deficiency resulted in minimal increase in *Plasmodium chabaudi* parasitemia (Taylor et al., 2001). Thus, further studies of these models
are needed to fully understand the role of complement activation in parasite growth.

5. Conclusions

We have demonstrated that merozoites are capable of invading RBCs via a previously unrecognized antibody and complement-medi- ated pathway by binding to CR1. Our findings are especially relevant to the efforts of developing an anti-merozoite malaria vaccine since some antibodies may actually enhance RBC invasion. Based on our findings we propose a model where the presence of anti-merozoite antibodies can activate complement and lead to binding to and aggregation of RBC CR1 (Fig. S16). The absence of complement could artificially en- hance the inhibitory activity of some antibodies. Thus, complement ac- tivity should be included in GIA to measure the inhibitory activity of anti-merozoite antibodies.

Funding Sources

This work was funded by Grant P131040 from the Congressionally Directed Medical Research Program, PI José A. Stoute. The funding agency played no role in the collection or interpretation of the data, the preparation of the manuscript, and the decision to publish.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Authorship Contributions

SB and JAS designed all the experiments. SB, JAS, and MEL carried out all the experiments. MDS, CFO, and EA carried out the malaria vaccine studies and provided the human serum samples.

Acknowledgments

We thank Dr. Henry Marsh, Celldex Therapeutics, for the gift of soluble CR1.

We are grateful to Mr. Wade Edris and the microscopy core facility at Penn State College of Medicine for assistance with confocal microscopy. We thank Nate Sheaffer, Jade Vogel, and Joseph Bednarzyk from the Penn State Hershey Flow Cytometry Core Facility for assistance with flow cytometry acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.05.015.

References

Angov, E., Aufiero, B.M., Turgeron, A.M., Van, H.M., Ockenhouse, C.F., Kester, K.E., Walsh, D.S., McBride, J.S., Dubois, M.C., Cohen, J., et al., 2003. Development and pre-clinical analysis of a Plasmodium falciparum Merozoite Surface Protein-1 (42) malaria vaccine. Mol. Biochem. Parasitol. 128, 195–204.

Araujo, M.N., Leser, P.G., Gabriel, J.A., Assaad, R.L., Atra, E., 1991. A simple radial immunodiffusion assay for the measurement of functional complement C2 activity. Braz. J. Med. Res. 24, 49–57.

Anumugam, T.V., Tang, S.C., Lathia, J.D., Cheng, A., Mughal, M.R., Chigurupati, S., Magnus, D.S., McBride, J.S., Diggs, C.L., Holder, A.A., Long, C.A., et al., 2005. The clinical-grade 42-kilodalton fragment of merozoite surface protein-1 of Plasmodium falciparum strain FVO expressed in Escherichia coli protects Aotus nancymai against challenge with homologous erythrocytic-stage parasites. Infect. Immun. 73, 287–297.

Datta, P.K., Rappaport, J., 2006. HIV and complement: hijacking an immune defense. Biomed. Pharmacother. 60, 561–568.

Davies, K.A., Hird, V., Stewart, S., Sivapalensamy, B.G., Jose, P., Epenetos, A.A., Walport, M.J. 1990. A study of in vivo immune complex formation and clearance in man. J. Immunol. 144, 4613–4622.

Fearon, D.T. 1980. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leucocyte, B lymphocyte, and monocyte. J. Exp. Med. 152, 20–30.

Ghiron, I., Barbashov, S.F., Klickstein, L.B., Tas, S.W., Jensenius, J.C., Nicholson-Weller, A., 2007. A complement receptor 1/CD35 is a receptor for mannan-binding lectin. J. Exp. Med. 192, 1797–1808.

Guevara Patino, J.A., Holder, A.A., McBride, J.S., Blackman, M.J., 1997. Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. J. Exp. Med. 186, 1681–1689.

Harboe, M., Mølnes, T.E., 2008. The alternative complement pathway revisited. J. Cell. Mol. Med. 12, 1074–1084.

Huang, M., Uhrund, G., Vie, L., Fung, M., Mølnes, T.E., 2004. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin. Exp. Immunol. 138, 439–446.

Hartung, H.P., 2008. Advances in the understanding of the mechanism of action of Nwg. J. Immunol. 181 (Suppl. 3), 3–6.

Haynes, J.D., Moch, J.K., Smoot, D.S., 2002. Erythrocyte malaria growth or invasion inhibition assays with emphasis on suspension culture GIA. Methods Mol. Biol. 72, 535–554.

Jarra, W., Hills, I.A., March, J.C., Brown, K.N., 1986. Protective immunity to malaria. Studies with cloned lines of Plasmodium chabaudi chabaudi and P. berghei in CBA/Ca mice. II. The effectiveness and inter- or intra-species specificity of the passive transfer of immuinity with serum. Parasite Immunol. 8, 239–254.

Kaslow, D.C., Hui, G., Kumar, S., 1994. Expression and antigenicity of Plasmodium falciparum merozoite surface protein (MSP1(19)) variants secreted from S. cerevisiae. Mol. Biochem. Parasitol. 63, 283–289.

Kennedy, M.C., Wang, J., Zhang, Y., Miles, A.P., Chitsaz, F., Saul, A., Long, C.A., Miller, L.H., Stowers, A.W., 2002. In vitro studies with recombinant Plasmodium falciparum apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. Infect. Immun. 70, 6948–6960.

Kennedy, A.T., Schmidt, C.Q., Harboe, M., Friedman, G.E., Tarnay, P., Griffin, P.R., Barlow, P.N., Crabbe, B.S., Cowman, A.F., Thom, W.H., 2016. Recruitment of factor H as a novel complement evasion strategy for blood-stage Plasmodium falciparum infection. J. Immunol. 196, 1239–1248.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418–420.

Lutz, H.U., Jelezarova, E., 2006. Complement amplification revisited. Mol. Immunol. 43, 2–12.

Mastellos, D.C., Cuny, G., Koh, P., Harboe, M., Ulvund, G., Vien, L., Fung, M., Mølnes, T.E., 2004. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin. Exp. Immunol. 138, 439–446.

Naghdifard, I., Stoute, J.A., Franklin, P., Zaffary, R., 2010. Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet 376, 216–220.

Nilsson, U.R., Funke, L., Nilsson, B., Ekdahl, K.N., 2011. Two conformational forms of target-surface antibodies. PLoS Pathog. 7, e1002112.

Srivastava, G., Sarin, S., 2002. Complement-mediated killing of Plasmodium falciparum erythrocyte stages in culture. J. Infect. Dis. 186, 1248–1256.

Vodicnik, E., Czajowska, A., Mura, D., Baggio, R., Zabotto, A., Pavan, A., Gianfelici, A., et al., 2013. Complement receptor 1 (CD35) is a receptor for mannan-binding lectin. J. Exp. Med. 210, 1423–1436.

Srivastava, G., Sarin, S., 2002. Complement-mediated killing of Plasmodium falciparum erythrocyte stages in culture. J. Infect. Dis. 186, 1248–1256.
Ogutu, B.R., Apollo, O.J., McKinney, D., Okoth, W., Siangla, J., Dubovsky, F., Tucker, K., Waitumbi, J.N., Diggs, C., Witte, J. et al., 2009. Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. PloS One 4, e4708.

Otsyula, N., Angov, E., Bergmann-Leitner, E., Korch, M., Khan, F., Bennett, J., Otieno, L., Cummings, J., Andagalu, B., Tosh, D. et al., 2013. Results from tandem Phase 1 studies evaluating the safety, reactogenicity and immunogenicity of the vaccine candidate antigens Plasmodium falciparum FVO merozoite surface protein-1 (MSP1(42)) administered intramuscularly with adjuvant system AS01. Malar. J. 12, 29.

Pouw, R.B., Vredevoogd, D.W., Kuipers, T.W., Wouters, D., 2015. Of mice and men: the factor H protein family and complement regulation. Mol. Immunol. 67, 12–20.

Ramos, T.N., Darley, M.M., Weckbach, S., Stahel, P.F., Tomlinson, S., Barnum, S.R., 2012. The C5 convertase is not required for activation of the terminal complement pathway in murine experimental cerebral malaria. J. Biol. Chem. 287, 24734–24738.

Ricklin, D., Hajasenggalis, C., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 11, 785–797.

Rosa, T.F., Flammersfeld, A., Ngwa, C.J., Kiesow, M., Fischer, R., Zigler, P.F., Skerka, C., Pradel, G., 2016. The Plasmodium falciparum blood stages acquire factor H family proteins to evade destruction by human complement. Cell. Microbiol. 18, 573–590.

Rotman, H.L., Daly, T.M., Chynes, R., Long, C.A., 1998. Fc receptors are not required for antibody-mediated protection against lethal malaria challenge in a mouse model. J. Immunol. 161, 1908–1912.

Schachareon, A., Burnouf, T., Ouattara, D., Attanath, P., Bouharoun-Tayoun, H., Chantavanich, P., Foucault, C., Chongsupsaikulwong, T., Durilhe, P., 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am.J.Trop. Med. Hyg. 45, 297–308.

Sagara, I., Dicko, A., Ellis, P.D., Faye, M.P., Druilhe, P., 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am. J. Trop. Med. Hyg. 45, 297–308.

Sahu, A., Kay, B.K., Lambris, J.D., 1996. Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. J. Immunol. 157, 584–591.

Schlesinger, L.S., 1993. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. J. Immunol. 150, 2920–2930.

Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R., Horwitz, M.A., 1990. Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. J. Immunol. 144, 2771–2780.

Siddiqui, W.A., Tam, L.Q., Kan, S.C., Kramer, K.J., Case, S.E., Palmer, K.L., Yamaga, K.M., Hui, G.S., 1986. Induction of protective immunity to monoclonal-antibody-defined Plasmodium falciparum antigens requires strong adjuvant in Aotus monkeys. Infect. Immun. 52, 314–318.

Singh, S., Kennedy, M.C., Long, C.A., Saul, A.J., Miller, L.H., Stowers, A.W., 2003. Biochemical and immunological characterization of bacterially expressed and refolded Plasmodium falciparum 42-kilodalton C-terminal merozoite surface protein 1. Infect. Immun. 71, 6766–6774.

Singh, S., Miura, K., Zhou, H., Muratova, O., Keegan, B., Miller, L.H., Saul, A.J., Miller, L.H., Long, C.A., 2006. Immunity to recombinant Plasmodium falciparum merozoite surface protein 1 (MSP1): protection in Aotus nancymai monkeys strongly correlates with anti-MSP1 antibody titer and in vitro parasite-inhibitory activity. Infect. Immun. 74, 4573–4580.

Spadafora, C., Awandare, G.A., Kopylovska, K.M., Cize, J., Moch, J.K., Finberg, R.W., Tsokos, G.C., Stoute, J.A., 2010. Complement receptor 1 is a sialic acid-independent erythrocyte receptor of Plasmodium falciparum. Plu5 Pathog. 6, e1000968.

Spring, M.D., Cummings, J.F., Okkenhaugen, C.F., Dutta, S., Reidler, K., Angov, E., Bergmann-Leitner, E., Stewart, V.A., Bittner, S., Juopmans, L. et al., 2009. Phase 1/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A. PLoS One 4, e5254.

Stubbs, J., Simpson, K.M., Triglia, T., Plouffe, D., Tonkin, C.J., Duraisingh, M.T., Maier, A.G., Winzeler, E.A., Cowman, A.F., 2005. Molecular mechanism for switching of P. falciparum invasion pathways into human erythrocytes. Science 309, 1384–1387.

Tax, S.W., Klickstein, L.B., Barbashov, S.R., Nicholson-Weller, A., 1999. C1q and C4b bind simultaneously to CR1 and additively support erythrocyte adhesion. J. Immunol. 163, 5056–5063.

Taylor, P.R., Seixas, E., Walport, M.J., Langhorne, J., Botto, M., 2001. Complement contributes to protective immunity against reinfection by Plasmodium chabaudi chabaudi parasites. Infect. Immun. 69, 3853–3859.

Tham, W.H., Wilson, D.W., Lopatiuk, S., Schmidt, C.Q., Tetteh-Quarcoo, P.B., Barlow, P.N., Richard, D., Corbin, J.E., Breeson, J.G., Cowman, A.F., 2010. Complement receptor 1 is the host erythrocyte receptor for Plasmodium falciparum PRRb4 invasion ligand. Proc. Natl. Acad. Sci. U. S. A. 107, 17327–17332.

Waki, S., Waki, S., Takai, T., Tagawa, Y., Iwakura, Y., Mizuguchi, J., Nariuchi, H., Tsokos, G.C., Stoute, J.A., 2010. Complement receptor 1 is a sialic acid-independent erythrocyte receptor of Plasmodium falciparum. Plu5 Pathog. 6, e1000968.

Ward, P.A., Sterzel, R.B., Lucia, H.L., Campbell, G.H., Jack, R.M., 1981. Complement does not facilitate plasmodial infections. J. Immunol. 126, 1826–1828.

World Health Organization, 2013. World Malaria Report 2013 (Geneva).