Turkey [17]. Therefore, comprehensive studies are needed to completely understand the immune responses to *P. vivax* antigens in this region. Such an understanding will facilitate the design of effective vaccination strategies against *P. vivax* [9].

As a first step, in the present study, we analyzed naturally acquired antibody responses to four *P. vivax* antigens that may be potential vaccine candidates. Total IgM and IgG antibody responses (including IgG subclasses) to three blood-stage antigens, PvMSP1$_{19}$, PvAMA1-ecto and PvSERA4, and one pre-erythrocytic stage antigen, PvCSP, were examined in individuals living in Sanliurfa. Any association between antibody responses to each of the *P. vivax* antigens and the degree of parasitemia in the patients was investigated. Baseline data provides important information for the elimination programs targeting vivax malaria by drugs or vaccines.

Results

Naturally acquired antibody responses to pre-erythrocytic and erythrocytic antigens

Although the vivax malaria elimination program was launched in 1925 [18], there is no clear information regarding the endemicity rates and transmission dynamics in southeastern Turkey. According to the WHO, *P. vivax* transmission was reported in seven Turkish provinces in 2006, and 84% of cases occurred in the southeastern cities of Diyarbakir and Sanliurfa [17]. The Sanliurfa region, which accounts for the majority of malaria cases, has recently been studied [16,17]. For the present study, 195 serum samples were collected from the towns of Siverek and Harran.

The naturally acquired immune responses to the four *P. vivax* candidate vaccine antigens (PvMSP1$_{19}$, PvAMA1-ecto and PvSERA4 and PvCSP) were examined and total IgM and IgG antibody responses (including IgG subclasses) were measured. In the total study population ($n = 195$), 79.1% individuals were seropositive for either IgG or IgM against at least one of the four antigens studied, while 62.1% were seropositive for IgG alone, and 65.6% were seropositive for IgM alone (Table 1). IgG responses to PvMSP1$_{19}$ were seen most frequently in 50.3% of individuals, followed by responses to PvCSP (33.8%), PvAMA1-ecto (21%) and PvSERA4 (16.4%). However, IgM antibody responses to PvSERA4 and PvCSP were observed in the majority of individuals (49.2% and 36.9%, respectively) (Table 1). The IgG subclasses observed in the IgG responders were mainly IgG1, followed by IgG3. The IgG3 responses to PvCSP were more prevalent than those to PvSERA4. In addition, IgG2 and IgG4 responses to PvSERA4 were seen more frequently (in 15.6% and 12.5% of patients, respectively) than to the other antigens (Table 1). Taken together, these results show > 60% IgG seroprevalence to tested *P. vivax* antigens in individuals living in the Sanliurfa region. Interestingly, despite the acknowledged lack of sensitivity in estimating malaria transmission using IgM responses [19], at the present data-set 35–50% of the samples showed IgM responses to at least one of the antigens (particularly to PvCSP and PvSERA4).

Antibody responses in relation to a prior history of malaria infection

Although 2000–6000 cases of malaria were recorded annually in Sanliurfa up until 2003, the incidence declined rapidly after the government's efforts to control the disease using chloroquine and primaquine [17]. According to the Annual Report of the Sanliurfa National Malaria Control Center, the incidence of parasite infection in 2002, 2004 and 2008 was 12.96, 3.20 and 1.85 per 1000 population, respectively [17], and the number of *P. vivax* cases decreased by about 86% from 2002 to 2008 and by 42% from 2004 to 2008. Based on this information that the infection rate had decreased dramatically over the last decade, we examined whether there may be any difference in the antibody responses between younger (< 6 years old) and older individuals. The study population was grouped according to their history of malaria infection (Figure 1a). The results showed that 10% of PP (parasite positive) individuals had a history of malaria infection, but this rose to 30.5% in PN (parasite negative) individuals ($\chi^2 = 11.195$; $P = 0.001$). Importantly, the mean age of PN individuals with a history of malaria infection was significantly higher than that of individuals with no prior history of infection (27.6±17.9 years *vs.* 16.7±14.9 years; $P = 0.001$, $t = -3.383$). Thus, these observations suggest that in this region, it appears that repeated exposure to malaria correlates with significantly higher percentage of PN individuals.

We next analyzed the naturally acquired antibody responses to the four candidate antigens according to each individual's infection history. Primary *P. vivax* infections evoked high IgG responses to all four antigens, both in terms of frequency and serum concentration in the PP group with no history of malaria infection (Figure 1b and 1c). In cases of re-infection (which occurred in 10% of the PP group; Figure 1a), neither the prevalence nor the level of the IgG responses to the four antigens changed (Figure 1b and 1c). It is, however, noteworthy that IgG antibody levels to PvSERA4 were either very low or undetectable after re-infection. In contrast, PN individuals with a history of malaria infection showed higher IgG responses to all four antigens both in terms of frequency and serum levels (*$P<0.05$ and **$P<0.01$; Figure 1b and 1c). However, there was a substantial IgG response, but a very low response to PvMSP1$_{19}$ and PvCSP in those PN individuals with no prior history of malaria infection.

Table 1. Anti-*P. vivax* antibody prevalence in Sanliurfa.

Antigen	Antibody responses (n = 195) Number (%)			IgG subclasses among IgG responders (%)			
	IgG	IgM	IgG+IgM	IgG1	IgG2	IgG3	IgG4
PvMSP1	98 (50.3%)	55 (28.2%)	105 (53.6%)	93.9	11.2	70.4	5.1
PvAMA1-ecto	41 (21.0%)	35 (17.9%)	66 (33.7%)	95	7.5	77.5	7.5
PvSERA4	32 (16.4%)	96 (49.2%)	106 (54.1%)	90.6	15.6	59.4	12.5
PvCSP (chimeric)	66 (33.8%)	72 (36.9%)	88 (45%)	76.9	6.2	86.2	16.9
Total	121 (62.1%)	128 (65.6%)	155 (79.1%)				

doi:10.1371/journal.pone.0028126.t001

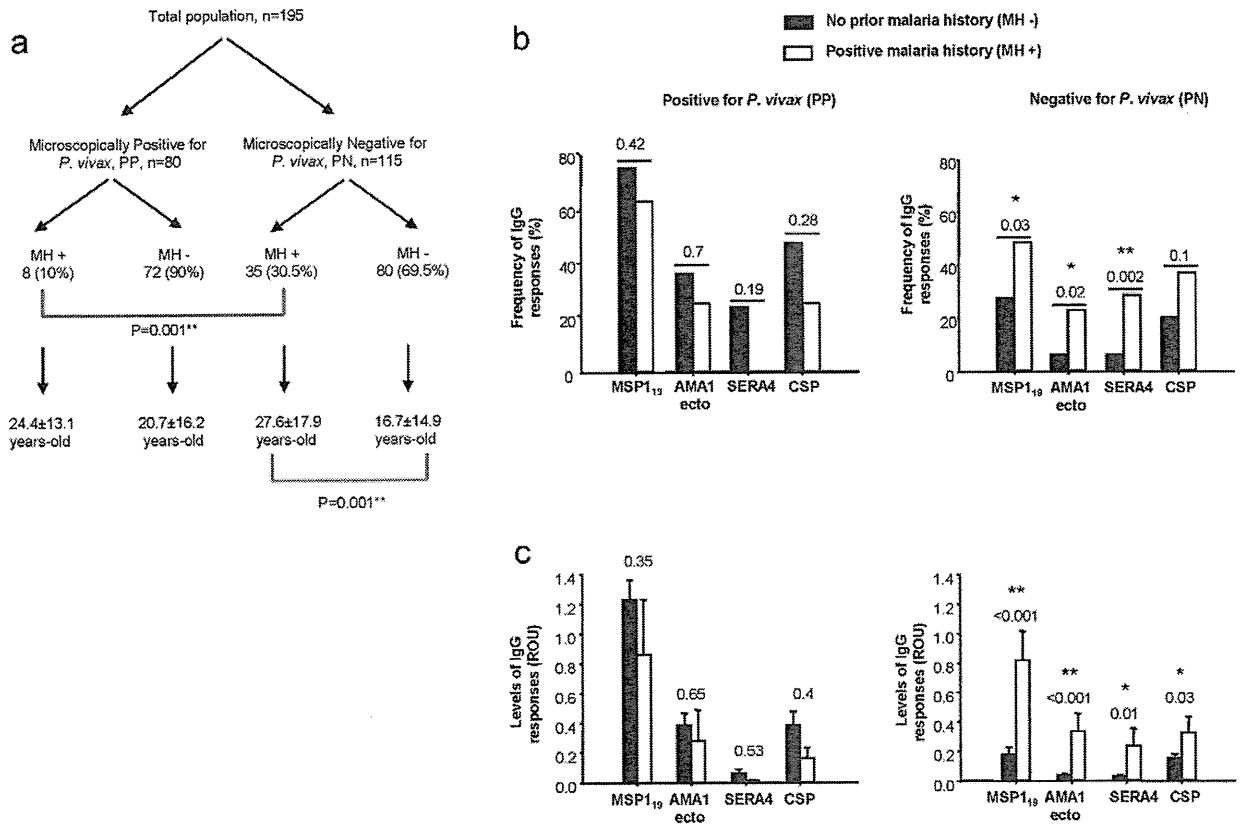

Figure 1. Impact of malaria infection history on naturally acquired antibody responses against *P. vivax* antigens. (a) Malaria infection history (MH) and its relationship to age. The total population was categorized according to blood smear positive (PP) and negative (PN) status for *P. vivax*, and then according to malaria infection history and age. The frequency (percentage seropositivity) **(b)** and serum antibody levels (ROU) **(c)** of the total IgG responses against the four antigens are depicted. The χ^2 test was used to compare the frequencies and mean ages between groups **(a)**. Bars **(c)** represent the mean±SEM, and ROU levels were compared using multiple comparison analysis in conjunction with ANOVA (*$P<0.05$ and **$P<0.01$).

doi:10.1371/journal.pone.0028126.g001

When combined, these results give important clues on the likely relationship of previous malaria exposure and seroconversion to malaria antigens tested.

Correlation between antibody frequency, serum concentration and age

We next examined the relationship between age and serum antibody responses, either between different age groups (Figure 2) or within the whole study population (Figure 3). With the exception of PvCSP, the frequency of IgG antibody responses to most of the antigens tended to increase with age (Figure 2a), while the serum levels did not differ significantly between the age groups (Figure 2c). Approximately 10–35% of individuals showed positive IgG antibody responses to at least one of the antigens by the age of 6, increasing to ~25–60% after the age of 30 (Figure 2a). For the whole study population, the trend of increasing total IgG responses with age was most marked for PvSERA4 ($r=0.322$; $P=0.0001$) (Figure 3). IgG3 responses to PvSERA4 and PvAMA1-ecto also increased significantly with age ($r=0.200$, $P=0.005$ and $r=0.282$, $P=0.0001$, respectively; Figure 3).

In contrast, there was no correlation between IgG responses to any of the antigens and age in the PP population ($n=80$; data not shown and Figure 2b). The increase in the IgG response was very rapid, even in the 0–6 age group, the exception being the IgG response to PvSERA4 (Figure 2b and Figure 2d). As expected, IgG responses (both in terms of frequency and serum levels) were lower in the PN population ($n=115$; Figure 2b and Figure 2d; $^{\#}P<0.05$ and $^{\#\#}P<0.01$; χ^2 test). However, despite the fact that IgG antibody responses were lower when compared with those in the whole study population or those in PP individuals (Figure 2d), 72% of PN individuals showed positive antibody responses (IgG and/or IgM) to at least one of the antigens (data not shown). Overall, IgG seropositivity against all the antigens studied (except PvCSP) in the PN population tended to increase with age (Figure 2b). The significant increase in the IgG response to PvSERA4 and the IgG3 response to PvAMA1-ecto with age (Figure 3) suggests that repeated exposure may be required to produce (and sustain) an increase in IgG responses to these two antigens. The IgG responses to most of the antigens (except PvSERA4) are apparent at a very early age; by contrast, IgG responses to PvSERA4 do not develop quickly after infection (s), but increases with age in concordance with PN status.

Correlation between antibody levels and parasitemia

We next evaluated the correlation between antibody responses and parasitemia during patent infection. Total IgG and IgG1 responses to PvCSP were positively correlated with parasitemia ($r=0.245$, $P=0.029$ and $r=0.246$, $P=0.028$, respectively;

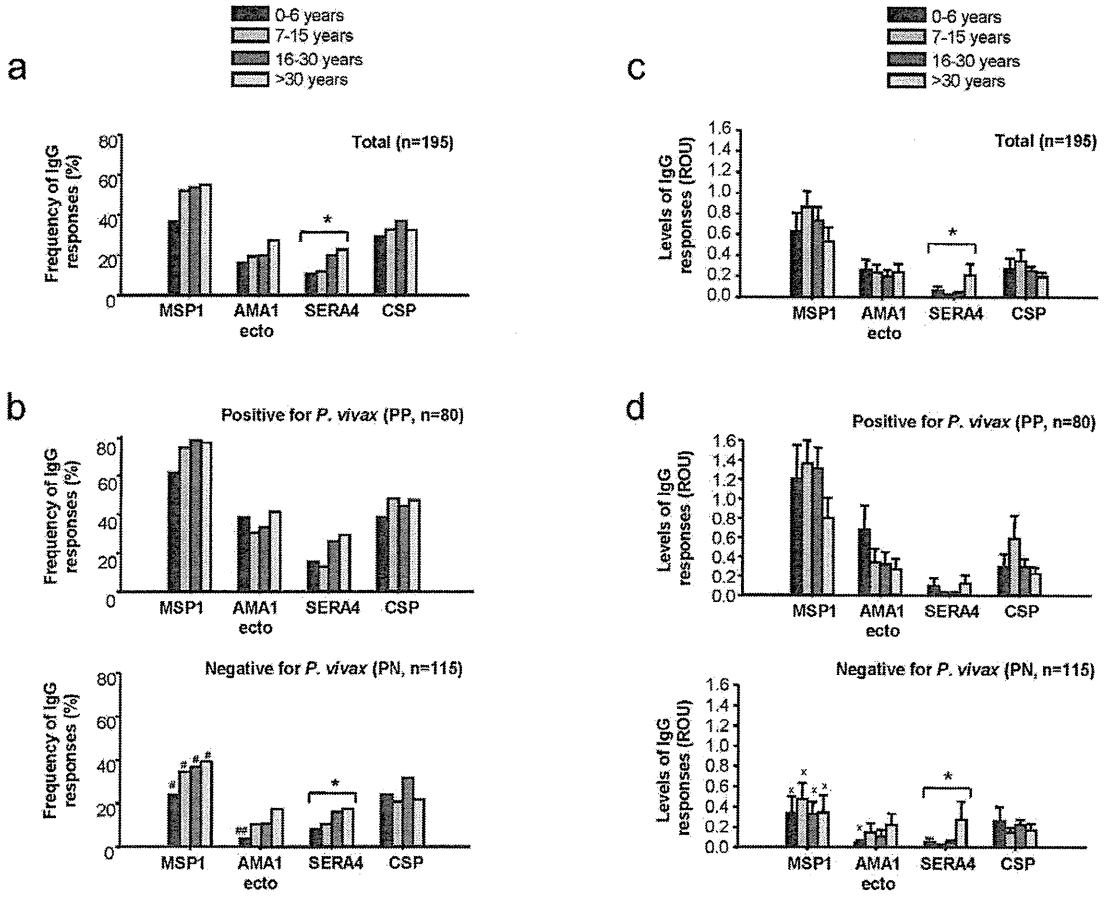

Figure 2. IgG antibody frequencies and levels according to age groups. IgG antibody frequencies in the total study population ($n = 195$) **(a)**, and in the blood smear positive (PP; $n = 80$) and blood smear negative (PN; $n = 115$) populations **(b)**. The number of individuals within each age group is shown in Table 1. # indicates significant differences between the PP and PN as assessed using the χ^2 test (#$P<0.05$, and ##$P<0.01$). Total IgG antibody responses (ROU) to the four antigens grouped according to age for the total population ($n = 195$) **(c)**, blood smear positive (PP; n = 80) and negative (PN; n = 115) individuals **(d)**. x indicates significant differences in antibody levels (ROU) between blood smear positive and negative groups using multiple comparison analysis in conjunction with ANOVA (x, $P<0.05$). * indicates a significant correlation between increases in antibody frequencies, levels and age ($r = 0.322$, $P = 0.0001$ and $r = 0.413$, $P = 0.001$ in **(a, c)** and **(b, d)** respectively).
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Figure 4). Conversely, IgG responses to PvAMA1-ecto were negatively correlated with parasitemia ($r = -0.233$, $P = 0.038$, Figure 4). There was no correlation between parasitemia and IgG3 levels for any antigen. These data support that PvCSP responses correlate well with parasite levels during acute infection [20]; however, the negative correlation between parasitemia and PvAMA1-ecto IgG responses may suggest an important role for high antibody levels to AMA1 in controlling parasitemia during *P. vivax* infection.

Discussion

Understanding immunity to malaria parasites is crucial for successful interventions. Despite the introduction of extensive malaria elimination programs since the 1920s [18], *P. vivax* malaria has re-emerged in southeastern Turkey. To date, no study has been performed to understand the epidemiology and transmission dynamics of the *P. vivax* parasites in this region. Southeastern Turkey is unusual in that individuals are infected with *P. vivax* alone, with no co-infection by other malarial species. Thus, analysis of this region has clear implications for *P. vivax* biology and developing various control strategies such as vaccines. Serological parameters were shown in *P. falciparum* infections to offer an advantage for measuring endemicity and malaria transmission dynamics, because of overcoming sampling variations and the detection of persistent antibodies over months and years after infection [21]. We used a similar approach to get an overview/rapid assessment of *P. vivax* transmission intensity. Likewise, this study is the first to analyze a range of serological parameters for individuals living in Sanliurfa, southeastern Turkey. Evaluations based on parasite prevalence, parasite exposure history and age may not be directly correlated with malaria protection but raise important clues for assessment in this unique malaria setting for *P. vivax* eventual monitoring and control.

The results of this study showed that IgG antibody responses to both pre-erythrocytic and erythrocytic antigens were closely related to a previous history of malaria infection. While only 10% of the actively infected population had a previous history of malaria infection, this increased three times (30.5%) in the PN population. This may indicate that one or two infections may induce naturally acquired immunity. Supporting this notion, IgG responses against *P. vivax* were positively correlated with age.

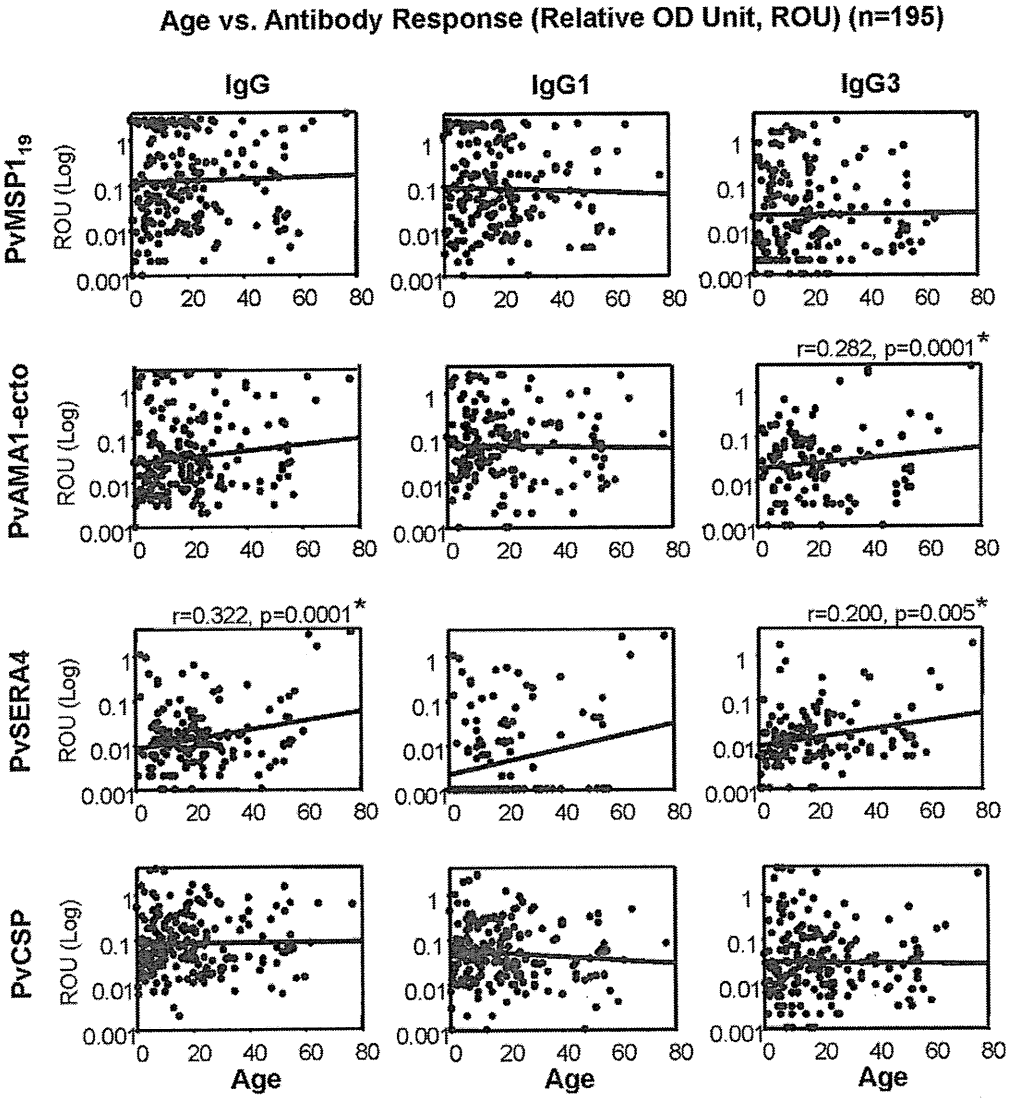

Figure 3. Correlation between age and total IgG, IgG1 and IgG3 antibody responses (ROU) to *P. vivax* MSP1$_{19}$, PvAMA1-ecto, PvSERA4 and chimeric PvCSP antigens in all individuals (n=195). Correlations were evaluated using Spearman's correlation test. Significantly correlated values were marked on the each figure (*).
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Importantly, antibody levels to PvSERA4 increased with age which might have implications for future vaccine design. In addition, the PvAMA1 ectodomain portion may be a potential candidate vaccine antigen for reducing parasite levels. Whether these observations can robustly correlate with malaria protection, a future study should be able to address these using defined cohorts.

In agreement with previously published studies of *P. vivax* and *P. falciparum* infection, IgG responses to PvMSP1$_{19}$ increased rapidly during the early ages of infection and were sustained over a long period of time [21,22,23,24], although there was no significant correlation between age, parasite levels, and antibody responses to PvMSP1$_{19}$. However, in an earlier pilot study, we observed higher sero-reactivity (53.6% IgG responses to PvMSP1$_{19}$) to the C-terminal region of the PvMSP1 antigen (produced in *Saccharomyces cerevisiae*) in the PP population, which was negatively correlated with parasite levels [16]. In the current study, we did not find any correlation between antibody responses to PvMSP1$_{19}$ and parasite number, suggesting the possibility of allelic polymorphisms in the antigen/parasites. In fact, in a recent study, we did find eight substitutions in the PvMSP1 gene that were unique to the Turkish *P. vivax* population and one of them (D/E at 1706 in the C-terminal 19-kDa region) was previously unidentified [17]. To understand whether this unique D/E substitution has impact on the antibody responses and parasite levels, we measured total IgG levels in a limited numbers of infected serum bearing the D/E substitution. Preliminary results suggested that there was an inverse correlation between anti-PvMSP1$_{19}$ IgG levels and parasite numbers in individuals infected with E haplotype substitutions, but not D haplotype substitutions (data not shown, *unpublished observation*). It is also noteworthy that other studies from Brazil and Papua New Guinea reported that different individuals

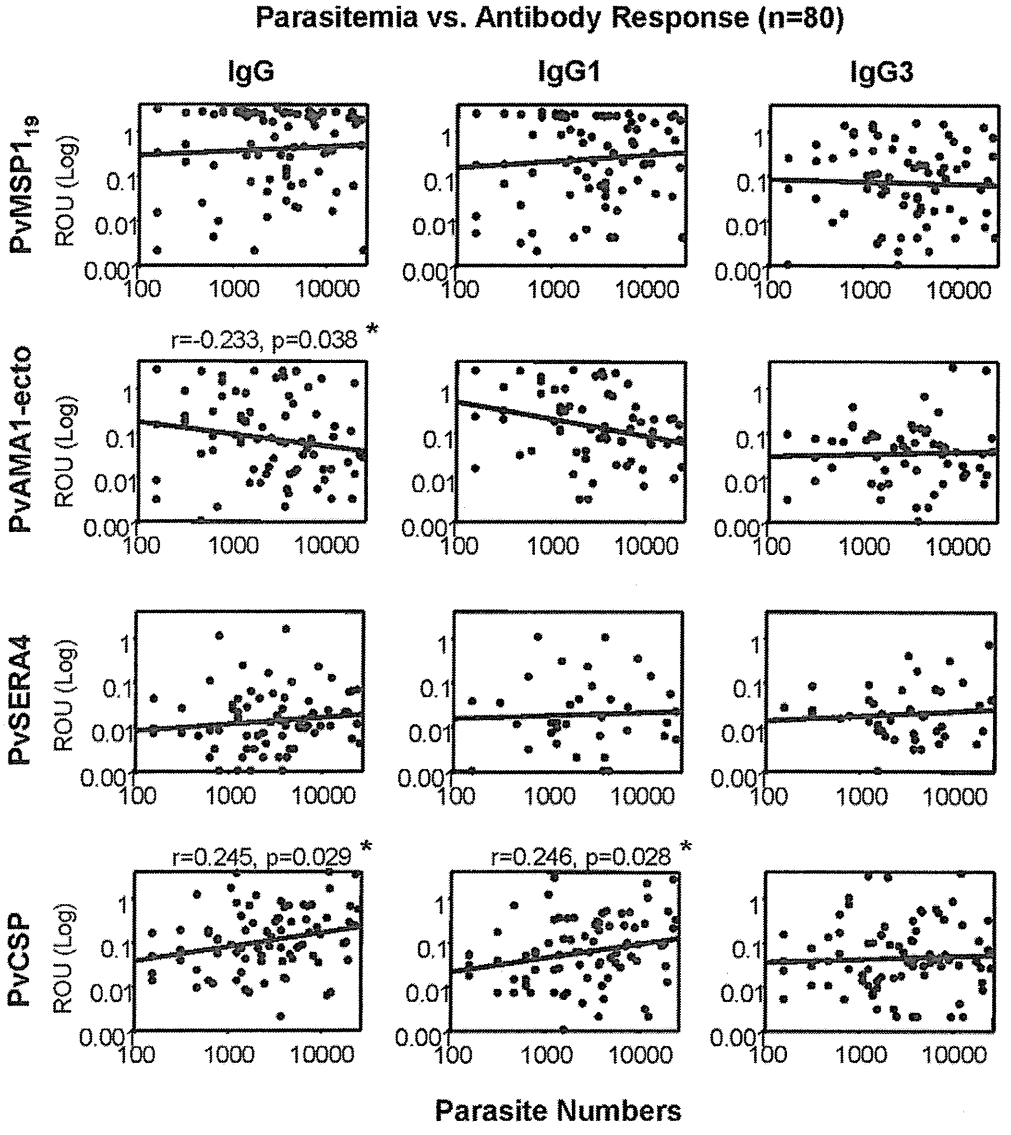

Figure 4. Correlation between parasite density and antibody levels. Total IgG, IgG1 and IgG3 antibody responses (ROU) to *P .vivax* MSP1$_{19}$, PvAMA1-ecto, PvSERA4 and chimeric PvCSP antigens were measured in individuals with patent *P vivax* infection ($n = 80$). Correlations were evaluated using Spearman's correlation test. Significantly correlated values are marked on each figure (*).
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show naturally acquired IgG responses to different regions of PvMSP1 [25,26]. Further confirmatory tests would be needed to substantiate further the effect of different haplotypes/allelic polymorphisms to antibody responses.

Similar to anti-PvMSP1$_{19}$ responses, antibody levels to chimeric PvCSP antigen immediately increased in younger individuals, but the response was weaker than that for PvMSP1$_{19}$, even during patent infection (Figure 2c). However, these responses did not completely disappear, and were even sustained in PN individuals with no evidence of prior malaria infection (presumably naïve; Figure 1c). One possible explanation for this might be an ongoing undetectable level of transmission by mosquitoes in this population as reported recently, suggesting the occurrence of *P. vivax* infection with ultra low-level parasitemia, which may maintain transmission levels, even under controlled-radical therapy [27]. Although we were technically unable to confirm the microscopically *P. vivax*-negative individuals by PCR in the present dataset, based on our experience from this region, genus-specific Real-Time PCR could only detect 4.5% parasite positivity over microscopy even in the malaria-suspected symptomatic patients (Yuksel and Zeyrek, *unpublished observations*). Therefore, it seems unlikely that more than 4.5% of malaria history negative PN group (presumably naïve) individuals have undetectable level of parasitemia. We cannot exclude, however, another possibility of *P. vivax* infections causing relapses due to the presence of hypnozoites, even in the absence of mosquito bites, because of ineffective drug treatment [28,29]. To date, it has always been a technical difficulty to address how hypnozoites/ relapses could influence serological profiles [30]. Currently there is no molecular tool to discriminate re-infection versus relapse during *P. vivax* infection and this certainly limits

meaningful evaluations to specific populations as re-infection probabilities may exceed 20% per year [31]. All together, these possibilities may explain the higher IgG seropositivity in parasite and presumably malaria history negative individuals.

We observed antibody responses to PvAMA1-ecto antigens in this region, although low in frequency and accompanied by low serum antibody levels. Children infected at a younger age (<6 years old) developed rapid antibody responses to AMA1-ecto antigen, but these responses were not seen after 6 years of age. However, the current study provides important evidence that IgG antibody responses to PvAMA1-ecto domain are significantly increased in non-infected individuals with a prior history of malaria infection. In addition, the IgG levels to PvAMA1-ecto were negatively correlated with parasite levels, which collectively may suggest that antibodies to PvAMA1-ecto are important and maybe closely related to protection [32]. These observations certainly require additional defined-cohort studies to address antibody levels-protection correlations.

This is the first report analyzing anti-*P. vivax* SERA4 responses. The serine repeat antigen (SERA) is an abundant asexual blood-stage antigen primarily expressed by *Plasmodium* parasites during late trophozoite and schizont stages [33,34,35]. In the case of *P. falciparum*, upon schizont rupture theabundant Pf SERA gene family member, SERA5, is processed to yield a 47 kDa N-terminal, a 50 kDa central, an 18 kDa C terminal, and a 6 kDa domain [34,35]. The N-terminal 47-kDa domain of *P. falciparum* serine repeat antigen 5 (PfSERA5) has already been exploited as a potential candidate vaccine [34]. An epidemiological study in a malaria hyper-endemic area of Uganda revealed that naturally-induced IgG responses to the N-terminal 47 kDa domain were positively correlated with increased levels of protective immunity in adults [36]. In earlier studies with *P. vivax*, the highest transcription of PvSERA4 in all field isolates [33] parallels that of PfSERA5. This was our basis on the selection of the expressed region of PvSERA4, which was similar in amino acid sequence to the 47-kDa domain of PfSERA5. In this study, we observed IgM responses to PvSERA4 in 50% of individuals, while only 16% showed IgG responses. Given that higher IgG levels were more frequent in the PN population only after re-exposure to malaria and after certain ages, there is a possibility that seroconversion from IgM antibodies to IgG for anti-PvSERA4 antibodies, are somehow not occurring properly. This is an interesting hypothesis that needs further investigation. Similar to the limited $PvMSP1_{19}$ gene polymorphisms observed in this region [17], initial analysis of the PvSERA4 gene in Sanliurfa isolates also revealed limited polymorphisms at the N-terminal region when compared with parasite isolates from southeast Asia (Arisue N and Horii T, *unpublished observations*). However, the polymorphic nature of the recombinant PvSERA4 antigen used in the present study (and the other antigens to a similar extent) may also be one reason that we detected low IgG antibody responses in this region. This, too, requires further investigation.

This study constitutes a first seroepidemiological analysis/survey of antibodies to a variety of blood and pre-erythrocytic stage malarial antigens and provides valuable information in their relation to the age of the individuals living in a sole *P. vivax* endemic region. However, we are far from the conclusion that naturally acquired antibodies to these relatively small numbers of vivax proteins could establish malaria immunity and/or protection. Apparently, new tools such as protein microarrays which represent at least 20% of the parasite proteome are needed to evaluate naturally acquired antibodies which may be associated with naturally acquired vivax malaria immunity in this setting as similar to recently described study for falciparum malaria [37].

Nevertheless, our study offers valuable insights to those designing vaccines/drugs especially in the settings where elimination programs have been launched [38].

Materials and Methods

Ethics Statement

All samples were collected after written informed consent was obtained from the patients (or the parents of individuals under 18 years of age), prior to anti-malarial treatment when appropriate. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Authorization was obtained from the Turkish Ministry of Health, Sanliurfa Bureau, and ethical approval was obtained from the Research Institute for Microbial Diseases, Osaka University.

Study population and demographic characteristics

One hundred and ninety-five serum samples were collected from individuals in Sanliurfa province (the towns of Siverek and Harran) in southeastern Turkey during 2004 and 2008. The samples were collected at the peak of the malaria season (July to November) and kept at −20°C until use. All 69 serum samples collected in 2004 were found to be infected with patent *P. vivax* malaria (as diagnosed by microscopy) [16]. The unbiased, age and gender matched 126 serum samples from 2008 were collected during active surveillance (house-to-house screening) (Table S1). Of these serum samples, 11 (8.7%) were confirmed *P. vivax*-infected using thick blood smears after Giemsa staining. The level of parasitemia (asexual parasites/μL blood) was determined as previously described [15]. Statistical analysis showed no significant difference between the two patient groups in terms of population demographics and antibody responses ($n = 69$ from 2004 and $n = 11$ from 2008) (data not shown); and hence, the two sets of samples were grouped together as "microscopically positive for *P. vivax* parasites" ($n = 80$) (see Table S1).

The mean age of the sample population was 20.5±16.2 years [0–77 years] (mean ± SD [range]), and 47.7% of the subjects were male. The samples were divided into four age groups: 0–6 years old (19.5%), 7–15 years old (26.7%), 16–30 years old (33.3%), and >31 years old (20.5%). Table S1 shows the baseline characteristics of the study subjects. The study included individuals infected with *P. vivax* (*P. vivax*-positive (PP) according to blood smear results; $n = 80$) and individuals living in the malaria-endemic area but not infected with *P. vivax* at the time of sampling (*P. vivax* negative (PN) according to blood smear results; $n = 115$). The mean (mean ± SD [range]) age of the two groups was 21.1±16 [0–65] years and 20.4±16.5 [0–77] years, respectively, and 56.2% and 41.7% of the respective groups were male. There was a significant difference in body temperature between the PP and PN groups (37.9±0.522°C *vs.* 36.7±0.513°C, respectively; $P<0.0001$). The mean parasite density was 5502±6386 [160–25560] parasites/μL. The majority of the patients (46.3%) had parasite densities between 1001 and 5000 parasites/μL, whereas only 32.5% of the patients had heavy parasitemia (> 5000 parasites/μL) and 21.3% had very low parasitemia (< 1000 parasites/μL). Mean hemoglobin, hematocrit concentrations, and mean white blood cell counts did not vary significantly between the PP and PN groups (Table S1).

Determination of malaria infection history

Disclosure of malaria infection is compulsory in Turkey and intervention is controlled by the Turkish Ministry of Health, alongside local national malaria control centers, in accordance with the World Health Organization criteria for malaria diagnosis and treatment. Questionnaires filled out by the subjects or their

parents ascertained whether they were previously diagnosed and treated for malaria at any of the local malaria control centers. The answers were cross-checked against the records from the malaria control centers. The results showed that those individuals with a history of previous malaria infection had only a single exposure (> 95%).

Recombinant *P. vivax* antigens

PvMSP1$_{19}$, PvAMA1-ecto, and PvCSP were expressed using a wheat germ cell-free protein translation system (CellFree Sciences, Matsuyama, Japan) [39], whereas PvSERA4 was cloned and expressed in *E. coli* as a histidine tagged fusion protein. The PvCSP was designed as recombinant chimeric protein that presumably cover vivax parasite population globally as previously described in detail [40]. PvAMA1-ecto encompasses ectodomain of PvAMA1 (Glu$_{77}$ to Gln$_{484}$ based on the *SalI* sequence, PVX_ 092275). Both gene fragments were cloned into pEU-E01-His-TEV-N2 plasmid for the wheat germ cell-free system (CellFree Sciences).

PvSERA4 gene sequence corresponding to amino acid regions Val$_{19}$ to Lys$_{352}$ was amplified from gDNA of *SalI* after comparing sequences with NICA and Chesson. The N-terminal position and prediction of peptide cleavage site was determined using SignalP 3.0 [41]. The C-terminal position was determined by alignment with PfSERA5 N-terminal domain. After alignment and intron identification/localization, introns were spliced by overlapping PCR. The amplified fragment was ligated to pET-15b, and the resulting plasmid with hexa-His-tag was transformed to *E. coli* Rosetta-gami B (DE3) pLysS (Novagen, San Diego, CA). Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The recombinant protein was extracted into soluble fraction with BugBuster Master Mix (Novagen) and purified using a His GraviTrap (GE Healthcare). All antigens were stored at −80°C until assayed.

Measurement of serum antibody levels by ELISA

The levels of human IgG, IgM, IgG1, IgG2, IgG3, and IgG4 antibodies against PvMSP1$_{19}$, PvCSP, PvAMA1-ecto and PvSERA4 were measured by ELISA as previously described [42,43]. Briefly, 96-well microtiter plates (Maxisorb, Nunc, Denmark) were coated with either 0.5 or 1 μg/ml of each of the recombinant antigens in bicarbonate buffer and incubated overnight at 4°C. After blocking, serum samples (100 μl, diluted 1:100 in a PBS-Tween buffer containing 5% skim milk) were added to the wells. The serum dilution and the amount of coated antigen were confirmed in pilot experiments as optimal for the accurate measurement of antibody levels. After washing four times, the plates were incubated with horseradish peroxidase-conjugated secondary antibodies to human IgG, IgM and the four IgG subclasses (1:1000; Zymed, Carlsbad, USA) at room temperature for 2 h. The plates were washed four times and 3, 3′, 5-5′-tetramethylbenzidine (Sigma) was added to each well and incubated in the dark at room temperature. The optical density was then measured at 450 nm. Each sample was assayed in duplicate and each plate contained "blank" wells and control sera. The OD values were normalized according to cut-off value and expressed as relative OD units (ROU). Cut-off values were set at three standard deviations above the mean OD$_{450}$ measured using sera from 20 Japanese blood donors with no history of malaria exposure. After subtracting the cut-off values, any ROU measurements higher than 0.01 were considered positive. The cut-off values were as follows: anti-PvMSP1$_{19}$-IgG, IgM, IgG1, IgG2, IgG3 and IgG4: 0.094, 0.345, 0.052, 0.324, 0.022 and 0.367, respectively; anti-PvAMA1-ecto-IgG, IgM, IgG1, IgG2, IgG3 and IgG4: 0.145, 0.542, 0.204, 0.32, 0.064 and 0.059, respectively; anti-PvSERA4-IgG, IgM, IgG1, IgG2, IgG3 and IgG4: 0.03, 0.405, 0.019, 0.062, 0.0418 and 0.01, respectively; and anti-PvCSP-IgG, IgM, IgG1, IgG2, IgG3 and IgG4: 0.141, 0.775, 0.14, 0.41, 0.05 and 0.1, respectively.

Statistical analysis

Data from 2004 and 2008 were pooled and/or divided into two groups: blood smear-positive and blood smear-negative for *P. vivax*. The antibody levels in all age groups, and in the blood smear-positive and -negative groups, were compared using the X^2 test. Differences in antibody levels (expressed as ROU) between the groups were analyzed using multiple comparison tests in conjunction with ANOVA. Spearman's rank correlation was used to evaluate correlations between the variables. A P value < 0.05 was considered significant. All statistical analysis was performed using the SPSS statistical package (version 10.0; SPSS, Chicago, IL, USA).

Supporting Information

Table S1 Demographic characteristics of the study population. * Significant values were determined between microscopically confirmed parasite positive vs. parasite negative populations by Student's t-test and X^2- tests with a level of significance set at $P<0.05$. [a] Statistically significant by Student's t-test.
(DOC)

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Author Contributions

Conceived and designed the experiments: FYZ NP KT TH TT KJI CC. Performed the experiments: FYZ YF NP MY ST CC. Analyzed the data: FYZ CC. Contributed reagents/materials/analysis tools: FY MY NA KH ST KT TH. Wrote the paper: FYZ NP MY TT KJI CC.

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Targeting Sialic Acid Dependent and Independent Pathways of Invasion in *Plasmodium falciparum*

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Abstract

The pathology of malaria is a consequence of the parasitaemia which develops through the cyclical asexual replication of parasites in a patient's red blood cells. Multiple parasite ligand-erythrocyte receptor interactions must occur for successful *Plasmodium* invasion of the human red cell. Two major malaria ligand families have been implicated in these variable ligand-receptor interactions used by *Plasmodium falciparum* to invade human red cells: the micronemal proteins from the Erythrocyte Binding Ligands (EBL) family and the rhoptry proteins from the Reticulocyte binding Homolog (PfRH) family. Ligands from the EBL family largely govern the sialic acid (SA) dependent pathways of invasion and the RH family ligands (except for RH1) mediate SA independent invasion. In an attempt to dissect out the invasion inhibitory effects of antibodies against ligands from both pathways, we have used EBA-175 and RH5 as model members of each pathway. Mice were immunized with either region II of EBA-175 produced in *Pichia pastoris* or full-length RH5 produced by the wheat germ cell-free system, or a combination of the two antigens to look for synergistic inhibitory effects of the induced antibodies. Sera obtained from these immunizations were tested for native antigen recognition and for efficacy in invasion inhibition assays. Results obtained show promise for the potential use of such hybrid vaccines to induce antibodies that can block multiple parasite ligand-red cell receptor interactions and thus inhibit parasite invasion.

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Introduction

In *Plasmodium falciparum*, the causative agent of the most lethal form of human malaria, the cyclical bursting and invasion of erythrocytes is responsible for all the clinical manifestations of the disease [1]. Continued survival of the parasite in the human host requires successful invasion of merozoites into uninfected erythrocytes. This is an active and sophisticated process, and requires multiple steps of interaction among receptors on the red cell and parasite ligands [2]. *P. falciparum* has developed the ability to invade red cells using multiple parasite ligand-erythrocyte receptor interactions that have become known as alternative invasion pathways [3]. Various parasite proteins can fulfill similar roles in the invasion process and hence any successful malaria vaccine will have to target all alternative pathways of invasion.

Two major types of invasion pathways have been described in *P. falciparum*: a sialic acid (SA) dependent pathway and a SA independent pathway. Two families of parasite ligands have been implicated in these invasion pathways. Proteins of the Erythrocyte Binding Ligands (EBL) family are stored in the micronemes, after production in the endoplasmic reticulum, and include EBA-175 [4], EBA-140 [5,6], EBA-181 [7,8] and EBL-1 [9]. Glycophorins (GP) A [10], B [11,12] and C [13] have been identified as the receptors to which EBA-175, EBL-1 and EBA-140 bind, respectively. The receptor to which EBA-181 binds has yet to be identified, but has been found to be neuraminidase and chymotrypsin sensitive, and trypsin resistant [7]. As the glycophorins are the major sialylated proteins on the erythrocyte, these parasite proteins largely govern the sialic acid (SA) dependent pathways of invasion.

In contrast, the second family of parasite ligands that mediate invasion are the *P. falciparum* reticulocyte binding protein-like homologues (PfRHs), PfRH1, PfRH2a, PfRH2b, PfRH3, PfRH4, and PfRH5, and these act largely through SA independent pathways [14–16]. RH1 is an exception in this group as it has been found to interact with erythrocytes in a sialic acid dependent manner [17]. The erythrocyte receptors for these proteins remain unknown, except for RH4, which has been found to adhere to complement receptor 1 (CR1) [18].

During the process of invasion, merozoites are unprotected within the blood stream. They are exposed to circulating host immune factors and, in natural human populations, encounter a heterogeneous population of erythrocyte surface proteins. Whilst there is evidence that antibodies against native EBL and RH proteins can inhibit invasion [19], it has also been shown, however, that there is differential expression of these proteins [20–22]. This results

in phenotypic variation of the invasion profiles. Such variation provides the parasite with the ability to evade antibody-mediated immune mechanisms and to utilize those erythrocyte surface proteins immediately present in the host blood cells. It is unlikely that vaccines incorporating only a single blood-stage antigen will be sufficient to provide adequate protection against the severity of the disease seen in malaria endemic areas given the extensive diversity of *Plasmodium*'s invasion repertoire and the variability of the human immune response.

In an attempt to dissect out the invasion inhibitory effects of antibodies against ligands from both the SA dependent and SA independent pathways, we have used EBA-175 and RH5 as model members of each pathway. EBA-175 binds to GPA for invasion of erythrocytes, requiring both the sialic acid residues as well as the peptide backbone of GPA for successful binding [10]. EBA-175 and other EBA family genes contain six extracellular regions, of which only region II binds erythrocyte receptors [19,23]. Despite the expression of this ligand by all *P. falciparum* clones, the effect of antibodies to region II of EBA-175 on the invasion of erythrocytes is variable [19,24–26]. EBA-175 is used by a wide variety of parasite clones for invasion, and a recent paper [27] shows that antibodies specific for EBA-175 block erythrocyte invasion through the EBA-175/GPA pathway. The authors also reported that inhibition of parasite invasion by antibodies to region II of EBA-175 is not affected by polymorphisms occurring in region II. RH5, while being the smallest member of the PfRH family at 65kDa, appears to have a critical role in invasion as attempts to disrupt the gene have not been successful [2,28]. Unlike other genes encoding merozoite surface proteins, such as *Pfama1*, there have been few non-synonymous mutations observed so far in the gene encoding PfRH5 [28], and there have been no reports to date of any genetic diversity studies from natural parasite populations. In this study, we analyzed the individual and combined effects of antibodies against these two parasite ligands on invasion. Results presented in this study validate the use of a combination of these two ligands as a potential vaccine that would have broad activity against *P. falciparum*.

Results

Antigen expression and testing for function

The members of the PfRH protein family have no obvious domain structures, such as the region II cysteine-rich domains of the EBL family. We therefore decided to use the entire PfRH5 protein as the immunogen as additionally, differing reports of ability to inhibit parasite invasion were reported based on the region of PfRH5 used for immunization [2,16,29]. It was also important to determine that the recombinant proteins used for immunization were conformationally similar to the native parasite proteins. Thus, besides looking at the purity and stability of the products, we also assessed the binding ability of these recombinant proteins to erythrocytes. We chose to express RH5 in the wheat germ system on account of the multiple advantages afforded by it. It is a eukaryotic expression system that has no glycosylation machinery, thus mimicking native *Plasmodium* antigen expression. It can be established as a high-throughput platform (in 6 well robotic formats), resulting in total yields of ~2 mg protein overnight. There has been a high success rate of expression reported in literature [30,31] from genome-wide malaria, human, and plant projects. The successful expression of the full-length recombinant RH5 (hereafter rRH5), produced by the wheat-germ synthesis method was confirmed by the presence of a ~63 kDa product on SDS-PAGE gel after elution of the total protein preparation, as shown in Figure 1A, lane 1. This rRH5 antigen also binds to normal erythrocytes: this was determined by loading the eluate from the Ni column purification onto an SDS-PAGE gel, the presence of where the expected a ~63 kDa was visible (see Figures 1A, lane 2 and 1B). This confirms that this method of expression was effective at producing a functionally conformed antigen. As we propose that rRH5 would be a candidate to add to the current EBA-175 vaccine regime, we have used the same sub-fragment of the EBA-175 antigen encompassing region II (EBA_175_{RII}) that is being utilized in clinical trials (Figure 1C). Thus, although correct expression of this recombinant has been determined elsewhere [32], we also have independently determined its conformity by erythrocyte binding, resulting in the expected ~80 kDa product, as shown in Figure 1D.

Anti-$EBA\text{-}175_{RII}$ and anti-rRH5 antibodies recognize native parasite protein

Mice were immunized with full-length rRH5, or $rEBA\text{-}175_{RII}$, or a combination of both, and ascites and cardiac bleed sera were obtained at the end of the immunization regimen. It is important to ensure that the sera produced recognize and react with the specific native parasite proteins to ensure any effect mediated by antibodies contained therein is specific to the immunogen. Therefore, all sera produced were assayed on both Western Blots of parasite extracts as well as in IFAs on smears of mature stage parasites. Immunoblotting with sera obtained on native parasite

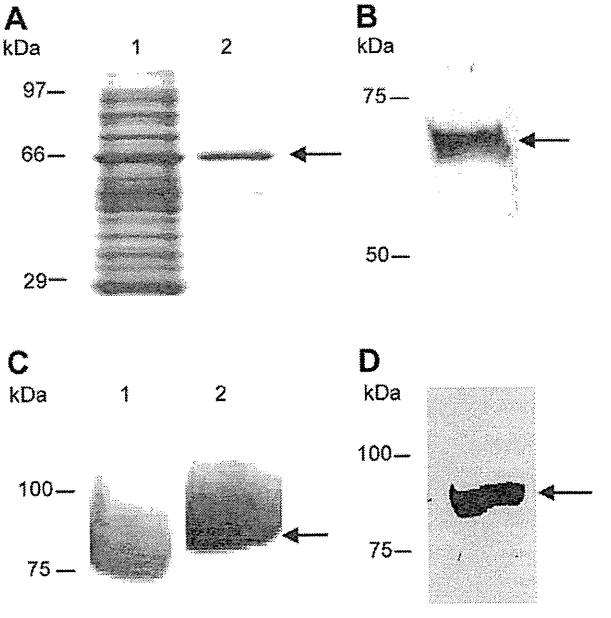

Figure 1. Recombinant EBA-175 and RH5 antigens are stable, pure and expressed in the correct conformation. The non-reduced (Lane 1 of panel **A**) and the reduced elution (Lane 2 of panel **A**; both visualized by Coomassie staining) of region II of EBA-175 synthesized using the yeast expression system *Pichia pastoris*, and the binding of this recombinant to normal erythrocytes (panel **B**), confirm correct expression and conformation of the $EBA\text{-}175_{RII}$ antigen with the expected product of ~80 kDa (indicated by the arrows in Lane 2 of panel **A** and panel **B**). The elution of full length RH5 synthesized using the wheat-germ synthesis (panel **C**, indicated by the arrow), and binding of rRH5 (panel **D**) to normal erythrocytes indicates functional conformity of this recombinant antigen, as shown by the presence of a single product at the expected size of ~63 kDa (indicated by arrows in both **C** and **D**).
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lysate (3D7) showed the anti-EBA-175$_{RII}$ antibody recognized native EBA-175, as determined by the 175 kDa product on SDS-PAGE gel (Figure 2A, lane 1). Immunoblotting with antibodies against the rRH5 protein showed that these antibodies are also specific for the native RH5 protein (Figure 2B). When sera from mice immunized with the combination of the two antigens, both a 175 kDa product and the native RH5 protein of 65 kDa are obtained (see Figure 2A, lane 2). The specificity of anti-EBA-175$_{RII}$ and anti-rRH5 to native protein was independently confirmed by immunofluorescence, using FITC-conjugated anti-mouse IgG and DAPI staining, and further confirmed that native EBA-175 and RH5 are localized to the apical end of merozoites, as expected for parasite proteins involved in invasion (see Figures 2C–2D).

Confirmation that anti-EBA-175$_{RII}$ is effective at inhibiting invasion of the parasite 3D7 isolate

After purifying the IgG fraction from the different sera, invasion inhibition assays (IIAs) in 250 μL cultures were established with total antibody concentrations ranging from 1.0 μg/mL to 500 μg/mL. All cultures were initiated at 0.8% parasitaemia using mature parasites (schizonts) purified off a Percoll gradient, and the resulting parasitaemia after 24–30 hours (i.e. after only one round of invasion) was determined. A dose-dependent curve of inhibition was obtained using IgG from EBA-175$_{RII}$ immunized mice and the degree of inhibition increased from 15%, at 1 μg/mL, to 84%, 500 μg/mL, compared to the no IgG control (Figure 3A). Inhibition assays performed with control IgG in this same range of concentrations yielded 5.0% to 23% inhibition. This confirms the specificity of the inhibition. However we observe a plateauing effect at 250 μg/mL, when the effective inhibition reaches 77% inhibition (see Figure 3A) and concentrations higher than this do not yield significantly higher inhibition. Previous studies with antibodies produced against the same EBA-175$_{RII}$ antigen in rabbits have shown up to ~80% growth inhibition [32,33], suggesting that this antigen is an effective immunogen, and provides encouragement for continuing with this antigen in human studies. Of note however, when this same EBA-175$_{RII}$ antigen was used to immunize

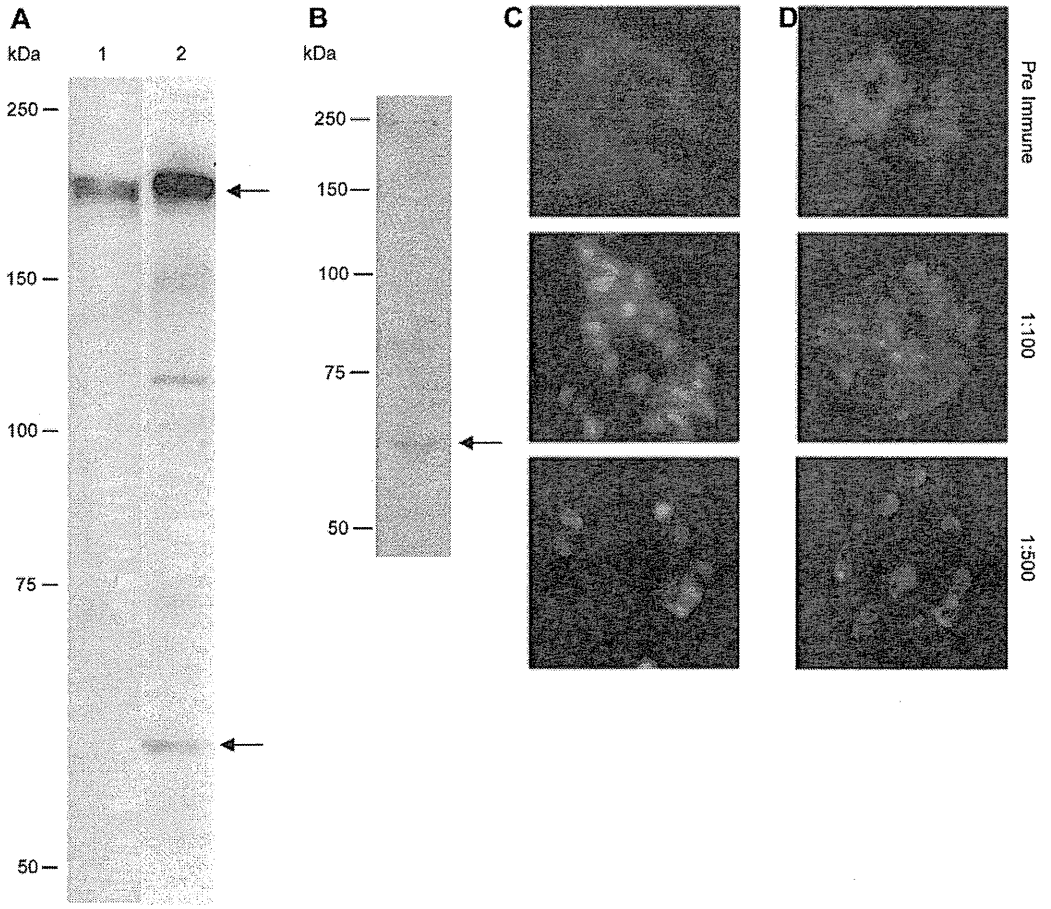

Figure 2. Recognition of native proteins. Anti-EBA-175$_{RII}$ antibodies are able to detect native EBA-175 protein of 175 kDa from 3D7 parasite lysates (Lane 1, panel **A**). Anti-EBA-175$_{RII}$/rRH5 (sera from the combined vaccine) is able to detect both EBA-175 and RH5 native proteins from the same lysate (Lane 2, panel **A**; EBA-175 indicated by top arrow; RH5 indicated by bottom arrow). Anti-rRH5 antibodies are specific for the native 65 kDa RH5 protein from 3D7 parasite lysate (panel **B**; indicated by arrow). All products were visualized with ECL after immunoblotting. Immunofluorescent detection of EBA-175 and RH5 in mature and rupturing 3D7 schizonts. Parasites were labelled with anti-EBA-175$_{RII}$ (panel **C**) or with anti-rRH5 (panel **D**) at dilutions of 1:100 and 1:500, then the slides were incubated with FITC-conjugated anti-mouse IgG (green) and mounted with 10 mg/mL DAPI (blue). EBA-175 and RH5 are each localised to the apical end of merozoites. Non-staining with preimmune sera (1:100 dilution) confirm specificity of each antibody to its respective antigen.
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malaria-naïve adults, only a low level of growth inhibition was observed (10–20%) despite high antibody titers [34], suggesting the need for additional components in an invasion targeting vaccine.

Anti-rRH5 antibodies exert potent anti-invasion effects in 3D7

This is the first study to test the effect of antibodies against full-length rRH5 on invasion of *P. falciparum*, and we obtained potent degrees of invasion inhibition ranging from 20% at 1.0 μg/mL, to a maximum of 72% at 250–500 μg/mL (Figure 3B). There is no difference in the levels of inhibition obtained from anti-rRH5 or anti-EBA-175$_{RII}$ between 1 μg/mL and 50 μg/mL, although, at the higher concentrations (100 μg/mL to 500 μg/mL), the level of inhibition from anti-EBA-175$_{RII}$ does exceed that of anti-rRH5 ($P<0.05$, T-test). These data are the first to indicate that PfRH5 alone is an effective immunogen, capable of eliciting an effective inhibitory immune response in these mice.

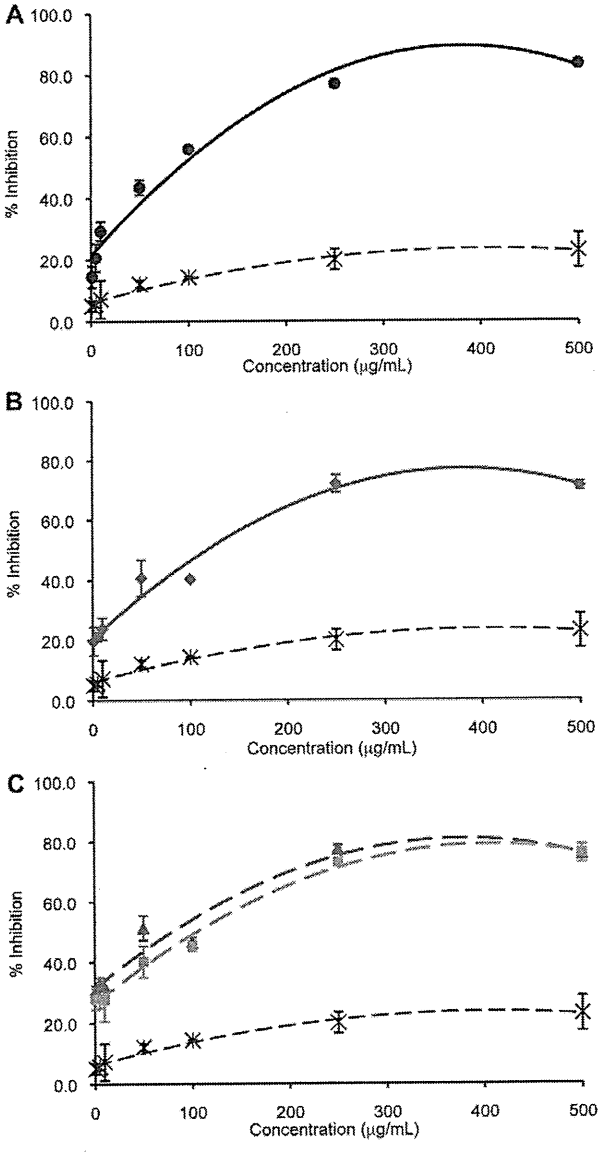

Figure 3. Invasion Inhibition of 3D7 with anti-EBA-175$_{RII}$ and anti-rRH5 antibodies. Anti-EBA-175$_{RII}$ (solid black line in panel **A**) and anti-rRH5 antibodies (solid blue line in panel **B**) inhibit invasion of 3D7 in a linear correlation to a similar extent. The two varying combinations used, anti-EBA-175$_{RII}$/anti-rRH5 and anti-EBA-175$_{RII}$+anti-rRH5 (solid red and solid green lines, respectively, in **C**), also showed the same positive correlation between increased antibody concentration and % inhibition (the color key for each antibody is conserved from **A**, **B**, and **C**). Percentage invasion inhibition from purified mouse IgG used as a control is shown as dashed black line (**A**, **B**, **C**).
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Combination anti-EBA-175$_{RII}$ and rRH5 antibodies show synergistic effects at the lower end of the concentration range

Apart from determining the inhibitory effects of antibodies to full-length RH5, the other purpose of this study was to look for synergistic effects on inhibition of invasion in the presence of antibodies to both EBA-175 and RH5, in order to block multiple ligands required for merozoite invasion simultaneously, thereby overcoming functional redundancy among invasion ligands and the capacity for immune evasion. Thus, IIAs were performed with two combination variants: anti-EBA-175$_{RII}$/rRH5 (obtained from immunizing mice with a single vaccine containing equal quantities of each antigen) and an artificial in-tube combination of the two IgGs after purification. This second combination used equal quantities of purified IgG from each individual serum, with 50% of the total IgG concentration coming from each antibody. As expected, there is a linear increase in invasion inhibition directly correlated to the increase in antibody concentration for both types of combination, and there is no significant difference between the level of inhibition obtained from these two types of combinations at any concentration ($P>0.05$, T-test; Figure 3C).

Interestingly, we observe two patterns of inhibition at the different ends of the concentration spectrum. At the lower concentrations of antibody (1.0 μg/mL – 5.0 μg/mL) we obtain a synergistic effect with the combination IgG a as these antibodies exert a greater inhibition on invasion than antibodies from the single immunogen sera used at the same concentration: 31% and 28% inhibition for the anti-EBA-175$_{RII}$/rRH5 and anti-EBA-175$_{RII}$+anti-rRH5, respectively, compared to 15% and 20% inhibition from the single anti-EBA-175$_{RII}$ and anti-rRH5 IgG, respectively ($P<0.05$, T-test for anti-EBA-175 *vs.* either combination; see Figure 4). As the concentration of antibody used in the IIA increases, however, this synergy is replaced by a different type of inhibitory profile where we obtain intermediate levels of inhibition to that observed for the single antibodies. Thus, at these concentrations (250 μg/mL to 500 μg/mL), the level of inhibition is equivalent to 50% contribution from anti-EBA-175$_{RII}$ plus 50% from anti-rRH5, suggesting that the individual components are acting independently to each other. At 100 μg/mL, the level of inhibition from each combination is only as great as anti-EBA-175, indicating that around 100 μg/mL, there is a transition from a synergistic effect to an independent effect. It is possible that when both antibodies are present and in excess, any synergistic effect is masked by some steric hindrance. If so, this would mean it is possible that as an individual antibody binds to its specific antigen, it creates a physical block for the other antibody to reach its antigen, thus the antigen is still available for binding to its erythrocyte receptor for invasion. We are currently investigating underlying mechanisms that could form the basis of this transition. Of interest to vaccine design and deployment, current studies indicate that titers of antibodies in vaccinated people would approximate the lower end of the concentration spectrum [34] and thus synergistic effects of antibodies from such combination vaccines would be expected to be obtained.

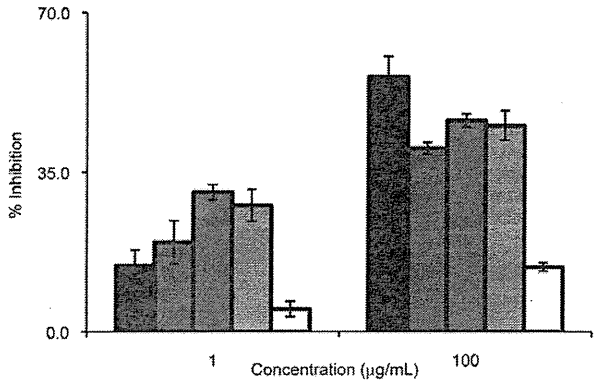

Figure 4. Anti-sera against the hybrid vaccine show synergistic effects at low concentrations. The invasion inhibition of both combination sera are greater than those obtained from the individual sera at the lowest concentrations used, 1 μg/mL. The combinations contain 50% of each individual immunogen (in the case of the combination vaccination, anti-EBA-175$_{RII}$/rRH5) or sera (in the case of the in-tube combination, anti-EBA-175$_{RII}$+anti-rRH5). However, by 100 μg/mL, the synergistic effects of the combinations are no longer apparent, and the inhibition from the combinations is equivalent to ~50% contribution from the two individual sera (anti-EBA-175$_{RII}$ shown by black bars, anti-rRH5 shown by blue bars, anti-EBA-175$_{RII}$/rRH5 shown by red bars, anti-EBA-175$_{RII}$+anti-rRH5 shown by green, control IgG shown by white bars).
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Inhibition of 3D7 parasite invasion in neuraminidase-treated erythrocytes confirms SA dependence of EBA-175 and SA independence of RH5

Treatment of erythrocytes with enzymes that selectively cleave moieties of malarial receptors would result in elimination of the use of that specific invasion pathway. We thus decided on the use of neuraminidase (Nm) that removes sialic acid residues from red cell receptors, such as GPA, the EBA-175 receptor. We reasoned this would eliminate the EBA-175 contribution to the invasion arsenal as its cognate receptor was impaired. Our hypothesis was confirmed when the invasion inhibition that was obtained in untreated erythrocytes in the presence of anti-EBA-175$_{RII}$ IgG effectively disappeared when Nm-treated cells were used in the invasion assay (Figure 5A). PfRH5, on the other hand, is known to bind to a non-sialylated receptor [2,16,28,29] and thus, as expected, we obtained a minimal difference in the invasion inhibition effect from anti-rRH5 between untreated and Nm-treated erythrocytes (Figure 5B). When the antigen combinations are used with Nm-treated cells, the expectation is that any invasion inhibition observed could only be from the action of the anti-rRH5 portion of the antibodies (the anti-EBA-175$_{RII}$ portion being blocked by the enzymatic treatment), and this is observed in the second combination when the independent antibodies are combined after purification: the invasion inhibition is reduced by ~60% from 46% in untreated cells to 28% in Nm-treated cells (Figure 5C). These data confirm that RH5 utilizes a SA independent pathway for invasion, as previously shown [16], and when the EBA-175/GPA pathway is blocked, 3D7 parasites will utilize this alternative, SA independent, pathway for invasion. However, there is no significant decrease from the anti-EBA-175$_{RII}$/rRH5 combination as expected (Figure 5C). This suggested that the RH5 portion of the combination vaccine was perhaps more immunogenic than the EBA-175$_{RII}$ component in these mice. However, titrating the sera against recombinant EBA-175$_{RII}$ and recombinant RH5 showed that both sera had approximately the same titers (~1:100,000, data not shown).

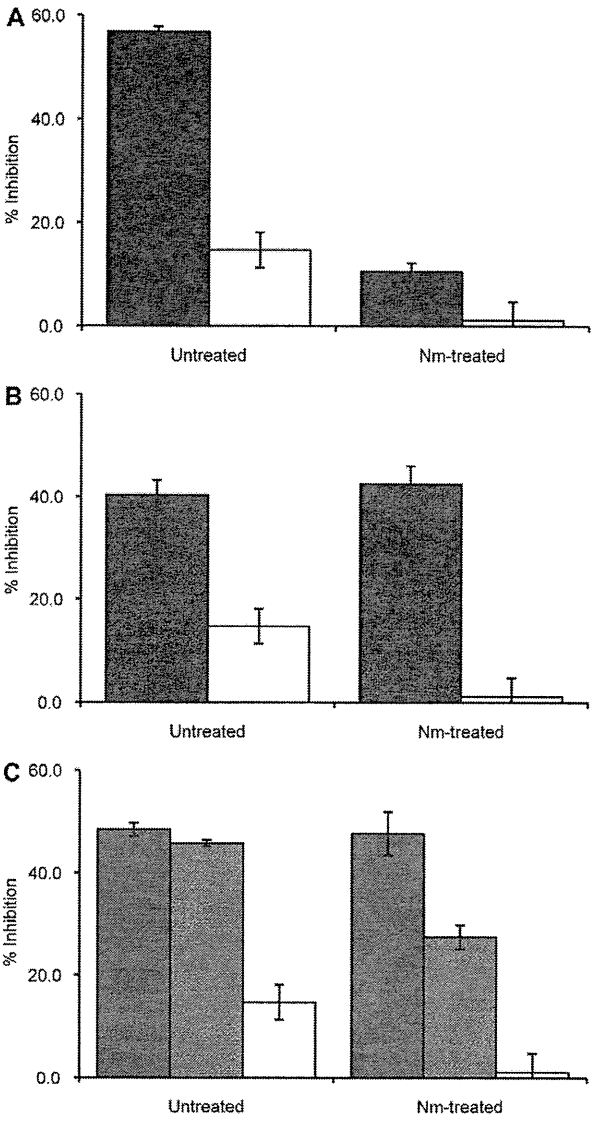

Figure 5. Enzymatic (neuraminidase) treatment of erythrocytes eliminates the inhibitory effects of anti-EBA-175$_{RII}$ antibodies but has only a mild effect on anti-rRH5 sera. Invasion inhibition assays with 3D7 and neuraminidase-treated cells (100 μg/mL antibody used) show that any inhibition due to the presence of anti-EBA-175$_{RII}$ antibodies is masked by the removal of ligands with sialic acid compared to untreated cells (**A**; anti-EBA-175$_{RII}$ and IgG shown as solid black and solid white bars, respectively). Anti-rRH5 alone (**B**; solid blue bars), or in combination (**C**; anti-EBA-175$_{RII}$/rRH5 and anti-EBA-175$_{RII}$+anti-rRH5 shown as solid red and solid green bars, respectively) is still able to significantly inhibit growth in treated cells as a sialic acid independent pathway is utilized by the RH5 antigen.
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Future work will be directed to understand this discrepancy between the two combinations.

Alternative pathways are used by the parasite for invasion

Different parasite strains use different erythrocyte receptors for invasion, such that some strains are totally dependent upon a

single pathway while others may have the ability to avail of multiple routes of invasion. It will be necessary that a malaria vaccine target all these different parasite strains. We thus decided to run the IIAs with the same set of sera on a parasite strain that has a very different invasion profile from 3D7 (SA independent). We chose Dd2 as it has been shown to be totally dependent upon GPA for invasion such that Nm-treatment of erythrocytes results in 100% inhibition of invasion in this strain [20], and the EBA-175/GPA pathway has been presumed to be the dominant SA dependent route of entry in this strain. Thus, we expected a high inhibition of invasion with Dd2 in untreated erythrocytes when anti-EBA-175$_{RII}$ antibodies are present: these antibodies will block the native EBA-175 antigen, preventing the EBA-175/GPA pathway required for invasion, mimicking the effect of Nm-treatment on erythrocytes. However, we observed no more than ~60% inhibition of invasion (at 100 μg/mL; Figure 6A). Although this is significantly greater than the inhibition seen with 3D7 at the same antibody concentration ($P = 0.015$, T-test), this is not a complete block as seen when Dd2 invasion is assayed in Nm-treated cells [35,36]. These results suggest that, contrary to published reports, alternative SA dependent pathways (i.e.EBL-1/GPB, EBA-140/GPC) are being utilized in Dd2 when the EBA-175/GPA pathway is not available. Further investigation using alternative SA dependent strains, such as W2-mef, are needed to elucidate this apparent paradigm. The inhibition profile when anti-rRH5 antibodies were used in the invasion assay are similar to those obtained with anti-EBA-175$_{RII}$ in that the level of inhibition at 50 μg/mL was equivalent to that of 3D7 (43% in Dd2, compared to 41% in 3D7; see Figure 6B). However, at 100 μg/mL, the inhibition observed was significantly higher in Dd2 than 3D7 (57% compared to 40%, respectively; $P = 0.003$, T-test). Interestingly, there was no difference in level of inhibition between the two strains for the anti-EBA-175$_{RII}$/rRH5 combination (antibodies from a combined vaccination) at either concentration (Figure 6C) but the level of inhibition observed in the presence of the anti-EBA-175$_{RII}$+anti-rRH5 (antibodies combined after purification) was significantly higher in Dd2 at both concentrations ($P<0.02$, T-test).

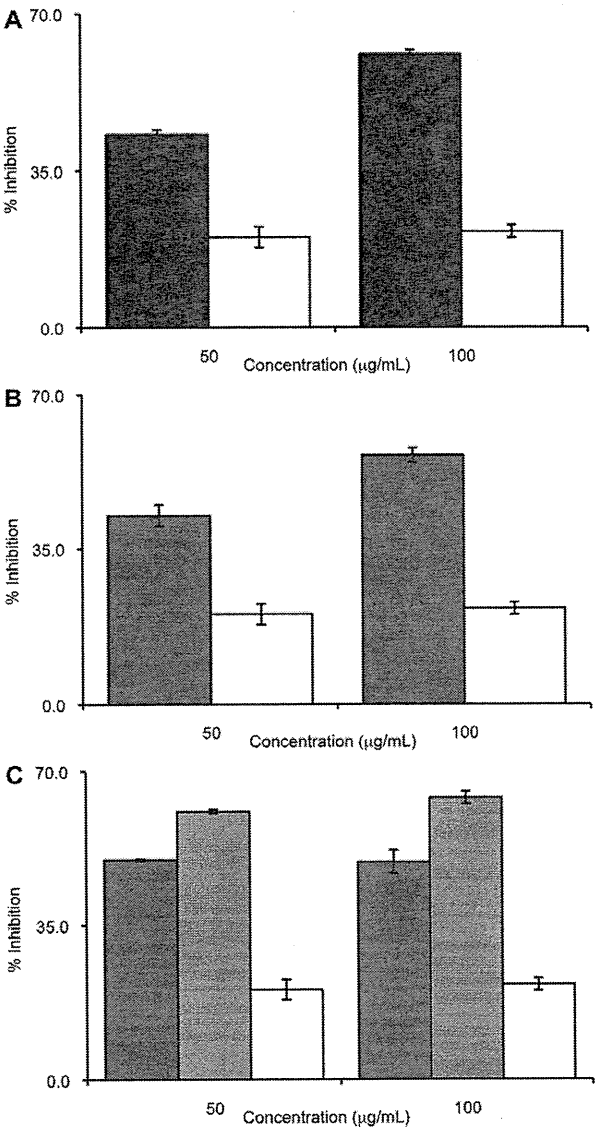

Figure 6. Dd2 parasites are not wholly dependent on the EBA-175/GPA pathway of invasion. In the presence of anti-EBA-175$_{RII}$ antibodies (**A**; black bars), inhibition when using the Dd2 strain is only 60% (Ab concentration of100 μg/mL), compared to 55% inhibition from 3D7, suggesting alternative SA dependent pathways are utilized by Dd2, such as EBL-1/GPB or EBA-140/GPC (for **A**, **B** and **C**, Dd2 with its control IgG shown as solid white bars). Although anti-rRH5 antibodies (**B**; blue bars), should only block a SA independent pathway, there is still greater inhibition with Dd2 compared to 3D7. In the presence of both antibodies (**C**; anti-EBA-175$_{RII}$/rRH5 shown as red bars and anti-EBA-175$_{RII}$+anti-rRH5 shown as green bars, respectively), there is a significant difference in the ability of Dd2 to invade compared to 3D7, especially when individual antibodies are combined.
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Discussion

To control and eventually eradicate malaria, an effective vaccine is considered to be necessary, in addition to currently existing tools, such as drugs and insecticide treated nets. The main requirement for the development of a successful anti-invasion malaria vaccine is the demonstration that antibodies made against each ligand can block the erythrocyte invasion of parasites. An *in vitro* parasite invasion inhibition assay (IIA) is one of the widely-used assays that can measure the functional activity of antibodies against asexual erythrocyte stages of *Plasmodium*. While it is still controversial whether the inhibitory activity measured by the IIAs reflects protective immunity induced by a malaria vaccine, the assay has been used in multiple preclinical and clinical studies as a prime immunological readout [27,37] and we have used it in this study to look at relative efficacy of two invasion ligands alone and in combination to elicit inhibitory antibodies.

EBA-175, specifically region II of this antigen, is currently a leading anti-malarial vaccine candidate, and EBA-175$_{RII}$ has been shown to be safe and immunogenic in malaria-naïve adults [34,38]. Further, the anti-sera from these vaccinated individuals inhibit, but do not eliminate, the growth *P. falciparum*, suggesting that additional components to the vaccine may be needed. As *P. falciparum* has been shown to invade erythrocytes by multiple pathways, using both the EBL and PfRH families a vaccine that is able to target more than one pathway is desirable. Recent studies have shown that the members of the EBL and PfRH families act cooperatively in merozoite invasion [37] and this has important implications for vaccine development. We chose to test EBA-175 in combination with PfRH5 as researchers have so far been unable to produce a PfRH5-knock-out, suggesting that this antigen is

essential for parasite invasion. Previous studies with RH5 report a mixed success with inducing invasion inhibitory antibodies that could be ascribed to the use of different regions of RH5 as immunogen [16,28,29]. So far, no studies have been done with full-length RH5 and to ensure a thorough validation of this ligand as a vaccine candidate, we undertook to assess the functionality and immunogenicity of the full-length RH5 protein alone and in conjunction with EBA-175$_{RII}$.

The rationale for including EBA-175$_{RII}$ in a multi-component vaccine has been well established by previous studies and these have shown that not only does this antigen elicit a suitable immune response, but anti-EBA-175$_{RII}$ antibodies are able to inhibit invasion of merozoites into erythrocytes by blocking the specific, SA dependent, EBA-175/GPA pathway [33] but by also inhibiting an alternative, SA independent, pathway [24,39]. Further, both the DNA and recombinant protein forms of the EBA-175$_{RII}$ vaccine component have been found to be safe and immunogenic in animal models and malaria-naïve human trials, respectively [25,26,34].

Our results with mouse anti-EBA-175$_{RII}$ antibodies show similar invasion inhibition in both 3D7 and Dd2 parasite strains and confirm the vaccine potential of this malarial ligand. A surprising find was that anti-EBA-175$_{RII}$ antibodies did not totally eliminate invasion of Dd2 as seen in its invasion of neuraminidase treated cells. The EBA-175/GPA pathway is thought to be the predominant route of entry for SA dependent parasite strains but this study shows that efficient invasion persists for this strain in the presence of potent anti-EBA-175 antibodies. Thus, other SA dependent pathways, EBL-1/GPB and EBA-140/GPC may be operating to overcome the loss of the GPA mediated pathway. Selection for these alternative ligand mediated pathways was not needed over time as was seen in the creation of the Dd2-Nm strain [35,36]. Further molecular analysis of gene expression of parasites in these IIA assays may shed light on the identity of these parasite ligands.

This is the first study to characterize the antibodies generated to full length RH5 and we report that the invasion-inhibitory potential of these antibodies equals that generated by EBA-175$_{RII}$ for both 3D7 and Dd2 parasites. Purified anti-rRH5 immunoglobulins recognized the native parasite ligand and inhibited merozoite invasion in a dose-dependent manner resulting in up to 72% inhibition at 500 μg/ml. These results indicate a possible reason for the inability to successfully delete the gene encoding RH5 from the parasite genome and stress the importance of determining the candidacy of the various EBL and RH ligands in such IIAs before designing rational vaccine combinations that will induce immune responses that ultimately may disrupt merozoite invasion.

The most interesting outcome of this study was the dual effect seen with the hybrid EBA-175$_{RII}$/rRH5 immunogen at either end of the concentration spectrum. The lower concentrations of antibody yielded the desirable "synergy effect" with the combination antigen inhibiting parasite invasion more efficiently than the individual components (additive effect). This effect dissipates at ~100 μg/ml and transitions to an inhibition profile that resembles a profile where each antibody seems to act independently (by 250 and 500 μg/ml), and the inhibition we obtain parallels that of the most active component. To understand this dual effect we have come up with a model that explains the outcomes based on whether the ligands (EBA-175 and RH5) act independently of each other or co-operatively. It would appear that when antibody is present at lower concentrations, the lack of steric hindrance allows the antibodies to exert an additive inhibitory effect (Figures 4 and 7). However, as the antibody concentration rises, the antibody against EBA-175 (the EBA-175 ligand is present in higher amounts than RH5 and also comes into contact with the red cell earlier (Ord et al, unpublished observations) saturates the surface of the erythrocyte not allowing anti-RH5 to mediate an effective inhibitory response (Figure 7). Thus, at higher concentrations of the combo antibody what is apparent in the IIA is only the inhibitory effect of anti-EBA-175$_{RII}$ IgG. This steric hindrance model may explain why the synergy seen with the hybrid immunogen at low concentrations disappears mid-range.

From the human vaccine point of view, this data is quite encouraging, as realistic titers from current human trials [34] indicate that they would approach the lower end of the IIA concentration spectrum used in this study. Thus, in the event of multiple antigen immunizations, synergistic effects among the antibodies to yield potent inhibitory effects on merozoite invasion may actually be the desirable outcome.

Methods

Ethics statement

All animal work in this study was carried out at A&G Pharmaceutical, Columbia, MD. The A&G Institutional Animal Care and Use Commitee reviewed and approved the animal protocols (protocol number AG-01) to ensure they met with strict accordance to the recommendations of the Guide for the Care and Use of Laboratory Animals of the NIH, and with accordance to the PHS Policy at A&G Pharmaceutical (OLAW AWA #A4404-01). Isoflurane was used to sedate the mice for immunizations, and all efforts were made to minimize suffering at all times.

Parasite culture

P. falciparum 3D7 and Dd2 lines were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) and cultured in human type A+ erythrocytes as described [40]. The identity of each strain has been confirmed by microsatellite fingerprinting [41].

Recombinant protein synthesis

Region II of the EBA-175 protein was synthesized by Cambrex Biosciences Inc., as described [34,42]. Full-length RH5 was produced by the wheat germ cell-free system (CellFree Sciences, Matsuyama, Japan) as described elsewhere [30,43]. Briefly, the full-length RH5 [comprising amino acid (aa) $_{26}$Glu to aa $_{526}$Gln of the 3D7 sequence without signal peptide, Met at N-terminus, and a hexa-histidine (HIS) tag at C-terminus] was cloned into the wheat germ cell-free expression vector, pEU-E01-MCS (CellFree Sciences) at XhoI/NotI sites. The recombinant RH5 protein, ~63 kDa with HIS-tag, was expressed using wheat germ cell-free system (CellFree Sciences) and purified using Nickel-Sepharose column (GE Healthcare, Camarillo, CA) as described previously [31].

Mouse immunizations/antibody production & purification

Balb/c mice in groups of 5, received one primary immunization and three boosts over a period of 2 months with either EBA-175$_{RII}$ alone, rRH5 alone, or an equal combination of both. Precision Antibody Proprietary Technology was used for all the immunizations. Ascites and cardiac bleed sera were obtained from all three regimes and pre-immune sera was collected from non-immunized mice. The IgG fraction from ascites sera were purified using Protein G sepharose beads (GE Healthcare) and dialyzed against 1x PBS overnight.

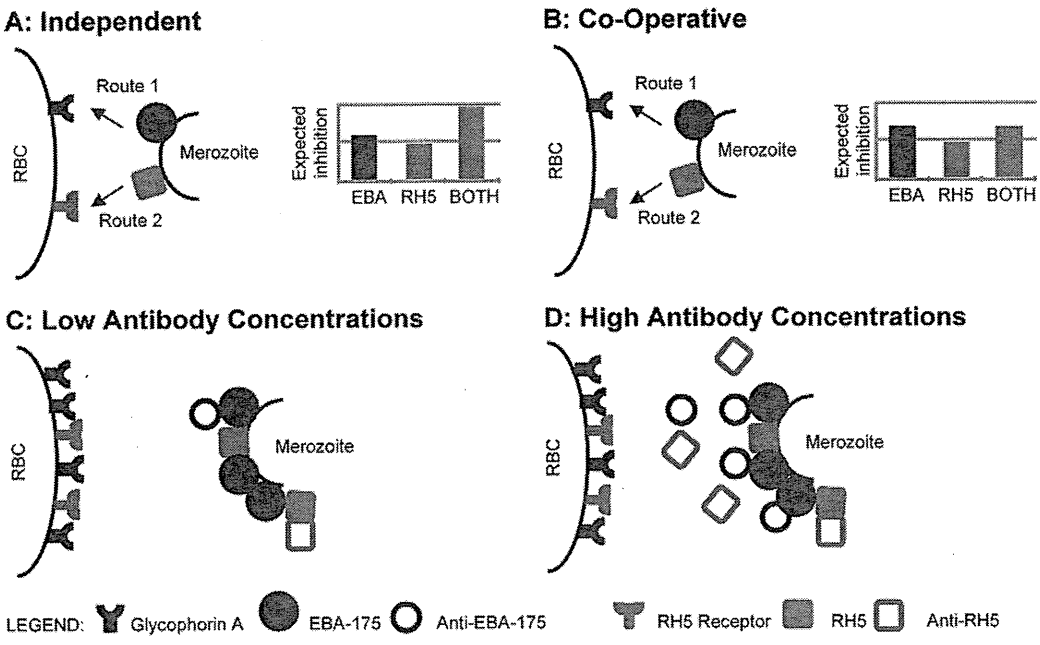

Figure 7. Independent Antibody Interaction and Co-Operative Antibody Interaction Models. ***Independent Antibody Interaction Model*** (Panel **A**): If the ligand-receptor interactions are independent of each other, invasion by either EBA-175 (Route 1) or by RH5 (Route 2) is not affected by the other. Thus, in the presence of both anti-EBA-175_{RII} and anti-rRH5 antibodies, the expected inhibition from the combination anti-sera is ADDITIVE (synergistic) when compared to the inhibition from the individual anti-sera. ***Co-Operative Antibody Interaction Model*** (Panel **B**): If the ligand-receptors act in a co-operative method, then invasion by EBA-175 (Route 1) and RH5 (Route 2) are not independent of each other. Thus, in the presence of anti-EBA-175_{RII} and anti-rRH5 antibodies, the expected inhibition from the combination anti-sera is only as effective as the most active individual antibody. ***Antibody Steric Hindrance*** (Panels **C** and **D**): Data suggests that EBA-175 abundance is greater than RH5 and is possibly released before RH5 (Ord et al, unpublished observations). At LOW antibody concentrations (**C**), there is no possible hindrance of RH5 by EBA-175, and all available antibodies are able to bind to their respective ligands independently. This is observed as growth inhibition with the combination anti-sera being more effective than that observed with the individual antibodies, i.e., it follows the Independent Antibody Interaction Model (**A**). Conversely, at HIGH antibody concentrations (**D**), anti-EBA-175 antibodies are able to bind to available EBA-175 ligands but they sterically hinder some RH5 antibody/ligand interactions, leaving some RH5 ligands available for invasion through the RH5 ligand/receptor pathways. This is observed as growth inhibition with the combination anti-sera being only as effective as anti-EBA-175_{RII} sera alone, i.e. it follows the Co-Operative Antibody Interaction Model (**B**).
doi:10.1371/journal.pone.0030251.g007

Erythrocyte binding assays

The recombinant EBA-175_{RII} and RH5 proteins were incubated with type A+ erythrocytes for 2 hr at room temperature. Erythrocytes were then washed with RPMI 1640 incomplete medium, layered over dibutylsulphate oil (Sigma), and centrifuged at 6,000 x *g* for 1 min. The supernatant and oil were removed by aspiration. Bound parasite proteins were eluted from the erythrocytes with 1.5 M NaCl and the eluate was used for immunoprecipitation/Western Blotting with 10 μg purified anti-EBA-175_{RII} or anti-rRH5 antibodies.

Immunoprecipitation/Western blotting

Saponin-lysed pellets from mature-stage parasites, or lysate obtained from erythrocyte binding assays, were separated under reducing conditions with either 6% or 10% SDS-PAGE gels, as appropriate. Anti-EBA-175_{RII} or anti-rRH5, along with appropriate secondary antibodies, were used to detect specific immunoreactivity. Mouse pre-immune sera was used as a control.

Immunofluorescence Assay (IFAs)

Mature schizont stage 3D7 parasites were smeared onto slides and stored at −70°C. Slides were thawed, fixed with 10% methanol/90% acetone for 20 min at room temperature. After air-drying, the smears were blocked and then incubated at room temperature for 1 hr with serum containing the anti-EBA-175_{RII}, anti-rRH5 or anti-EBA-175_{RII}/rRH5 antibodies, and then incubated with FITC-conjugated anti-mouse antibody (1:100) for 1 hr at room temperature protected from light. All slides were mounted using 10 μg/mL DAPI and observed under UV light. Images were merged using Adobe Photoshop Elements 6.0 software.

Neuraminidase treatment of erythrocytes

Cells were treated with 0.025 U/mL neuraminidase (*Vibrio cholerae*; Roche), at 37°C for 1 hr. The efficacy of each enzyme treatment was assessed in the Laboratory of Immunohematology, New York Blood Center, by assaying for loss of erythrocyte agglutinability, using a panel of monoclonal antibodies against suitable antigenic determinants on different blood group proteins.

Invasion inhibition assays (IIAs)

Mature trophozoite stages were isolated on 40%/70%/90% Percoll gradients as previously described [44]. Cultures for IIAs were established using a starting parasitaemia of 0.8% for 3D7 and 0.6% for Dd2, at a 5% haematocrit. Purified anti-EBA-175_{RII}, anti-rRH5, and combination anti-EBA-175_{RII}/anti-rRH5 antibodies were tested independently using 3D7 and IgG dilutions ranging from 1.0 μg/mL to 500 μg/mL. A second combination was also tested: equal proportions of anti-EBA-175_{RII} IgG and anti-rRH5 IgG were combined together after purification and

tested at final concentrations of 1.0 μg/mL to 500 μg/mL. Purified IgG from mouse sera (Sigma) was used at equivalent concentrations as negative controls. IIAs with Dd2 were performed at antibody concentrations of 1.0, to 100 μg/mL. IIAs at 100 μg/mL and 250 μg/mL with 3D7 were also performed on erythrocytes that had been treated with neuraminidase. All IIAs were assessed after 24–30 hr by enumerating infected erythrocytes on Giemsa stained smears. All IIAs were performed at least 3 times and the representative results from one such assay is shown in the figure.

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Author Contributions

Conceived and designed the experiments: RLO CAL. Performed the experiments: RLO MR TY ST. Analyzed the data: RLO MR TY ST TT CAL. Contributed reagents/materials/analysis tools: RLO MR TY ST TT CAL. Wrote the paper: RLO CAL.

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Article

PfSET10, a *Plasmodium falciparum* Methyltransferase, Maintains the Active *var* Gene in a Poised State during Parasite Division

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SUMMARY

A major virulence factor of the malaria parasite *Plasmodium falciparum* is erythrocyte membrane protein 1 (PfEMP1), a variant protein expressed on the infected erythrocyte surface. PfEMP1 is responsible for adherence of infected erythrocytes to the endothelium and plays an important role in pathogenesis. Mutually exclusive transcription and switched expression of one of 60 *var* genes encoding PfEMP1 in each parasite genome provides a mechanism for antigenic variation. We report the identification of a parasite protein, designated PfSET10, which localizes exclusively to the perinuclear active *var* gene expression site. PfSET10 is a histone 3 lysine 4 methyltransferase required to maintain the active *var* gene in a poised state during division for reactivation in daughter parasites, and as such is required for *P. falciparum* antigenic variation. PfSET10 likely maintains the transcriptionally permissive chromatin environment of the active *var* promoter and thus retains memory for heritable transmission of epigenetic information during parasite division.

INTRODUCTION

Malaria, an important infectious disease of humans, causes clinical cases in 300–500 million people and up to 1 million deaths per year. *Plasmodium falciparum* accounts for most of the burden of infection, and virulence is linked to the ability of infected erythrocytes to sequester in and obstruct the microvasculature of a variety of organs (MacPherson et al., 1985). Sequestration avoids destruction of the parasitized erythrocytes by the reticuloendothelial system and allows the microaerophilic parasite to mature in a relatively hypoxic environment (Raventos-Suarez et al., 1985). Moreover, this often leads to perturbation or complete obstruction of blood flow in the microcirculation. This abnormal circulatory behavior for red blood cells is related to parasite-induced alterations of adhesive properties and is key to the survival and pathogenicity of *P. falciparum* (Leech et al., 1984). Parasite-infected erythrocytes can adhere to platelets, vascular endothelial cells, and other erythrocytes (Barnwell, 1989; Berendt et al., 1989; Cooke and Coppel, 1995; Ockenhouse et al., 1992; Rogerson et al., 1995; Wahlgren et al., 1994).

The major virulence factor of *P. falciparum* is erythrocyte membrane protein 1 (PfEMP1), a protein expressed by the parasite on the surface of infected erythrocytes (Baruch et al., 1995; Su et al., 1995). This protein is responsible for adherence of *P. falciparum*-infected erythrocytes to receptors on endothelial cells and plays an important role in pathogenesis (Bull et al., 1998; Newbold et al., 1997). PfEMP1 proteins are encoded by approximately 60 *var* genes per genome and are located in subtelomeric and central regions of chromosomes (Gardner et al., 2002). Mutual exclusive transcription and switching to activate a different *var* gene provides the basis for antigenic variation and expression of ligands with differing receptor-binding properties (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995).

The *var* gene repertoire resides at the nuclear periphery in clusters of chromosomes (Freitas-Junior et al., 2000), where activation involves chromatin alterations (Voss et al., 2006) and repositioning of a specific *var* gene to a perinuclear location permissive for transcription (*var* gene expression site) characterized by histone marks H3K4me3 and H3K9ac and the presence of H2.AZ (Duraisingh et al., 2005; Dzikowski et al., 2007; Freitas-Junior et al., 2005; Howitt et al., 2009; Lopez-Rubio et al., 2007; Ralph et al., 2005; Petter et al., 2011), while repressed *var* genes are characterized by the conserved histone mark for heterochromatin, H3K9me3 (Lopez-Rubio et al., 2007).

The *var* gene promoter paired with the promoter found within the *var* intron are key elements in nucleation of transcriptional silencing, activation, and maintenance of allelic *var* gene exclusion (Dzikowski et al., 2006; Voss et al., 2006). The identity and molecular function of nuclear factors regulating chromatin modifications at the *var* promoter is not well understood. Although the *P. falciparum* genome contains a large repertoire of putative chromatin-modifying proteins, to date only a handful of nuclear factors have been characterized (Cui and Miao, 2010).

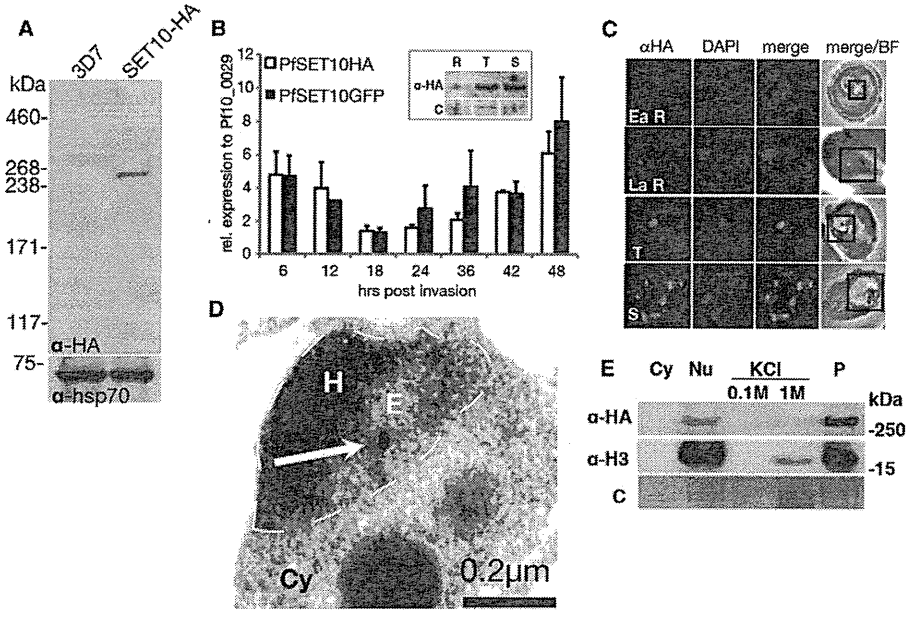

Figure 1. PfSET10 Localizes to a Compartment in the Nuclear Periphery

(A) PfSET10 (PFL1010c) was tagged with 3-HA epitopes, and anti-HA antibodies detected a 260 kDa protein. 3D7 served as control and Hsp70 as a loading control.

(B) Transcription analysis of PfSET10HA and PfSET10GFP in the intraerythrocytic life stages. Small inset shows PfSET10 protein expression in R, rings; T, trophozoites; S, schizonts. Data are represented as mean ± SEM of two independent experiments.

(C) PfSET10-HA was detected using anti-HA antibodies in IFA and shown to localize to a compartment of the nuclear periphery. First row, Ea R early ring; second row, La R, late ring; third row, T, trophozoite; fourth row, S, schizont; each row shows PfSET10 (green); DAPI staining (blue); PfSET10 and DAPI merged; PfSET10, DAPI and brightfield image.

(D) Immunoelectron microscopy localization of PfSET10 in 3D7SET10-HA schizonts using anti-HA antibodies. White arrow points to gold particles localizing with PfSET10. Nucleus is circled by a white dashed line. N, nucleus; Cy, cytoplasm.

(E) PfSET10 is located in the nuclear fraction. Shown are the cytoplasmic (Cy) and nuclear (Nu) fractions probed with anti-HA (upper panel) or anti-H3 antibodies (middle panel). Subsequent tracks show differential solubilisation of the nuclear fraction using 0.1 M and 1 M KCl and the insoluble fraction (P). The bottom panel shows a Coomassie-stained gel as a loading control. See also Figure S1.

This includes two silent information regulators (PfSirA and PfSirB) that play a key role in deacetylation of histone H3 and silencing of *var* genes (Duraisingh et al., 2005; Tonkin et al., 2009). Additionally, *P. falciparum* heterochromatin protein 1 (PfHP1) is a major component of heterochromatin in the perinuclear chromosome end clusters that binds specifically to H3K9me3 (Flueck et al., 2009).

Here, we report identification of a regulator of *var* gene activation in *P. falciparum* named PfSET10. PfSET10 was confined to a location in a euchromatic region of the nuclear periphery corresponding to the active *var* gene expression site. PfSET10 exhibits histone H3 lysine 4 methyltransferase activity, and our data indicate that PfSET10 is responsible for maintaining the *var* gene in the poised stage for re-activation in daughter cells.

RESULTS

*Pf*SET10 Localizes to a Site at the Nuclear Periphery

Previously, we identified a candidate regulator of *var* gene activation in *P. falciparum* (Volz et al., 2010), which we named PfSET10 (PFL1010c) and generated transgenic parasites by tagging the endogenous protein with GFP (Volz et al., 2010) or HA (Figures 1A, 1B and S1A). The HA-tagged PfSET10 had an approximate size of 260 kDa (Figure 1A). PfSET10 is transcribed throughout the intraerythrocytic life cycle, peaking during early and late stages (Figure 1B) in 3D7SET10-HA and 3D7SET10-GFP parasites (Figure 1B). Western analysis showed modest PfSET10 protein expression during ring stage compared to later stages (Figure 1B). This was supported by immunofluorescence assays (IFA) demonstrating no detectable PfSET10 protein in early ring stage but expression in a discrete perinuclear spot in each nucleus of later stage parasites (Figure 1C). Immunoelectron microscopy showed regions of electron-dense material at each nuclear periphery consistent with heterochromatin (Ralph et al., 2005), while PfSET10 localized to a heterochromatin-free region (Figures 1D and S1B). Subcellular fractionation of parasites confirmed PfSET10 was associated with nuclear chromatin (Figure 1E), similar to histone H3.

PfSET10 Localizes to the *var* Gene Expression Site

Current evidence suggests that *var* gene activation involves translocation of the locus to a specific region at the nuclear periphery (Duraisingh et al., 2005; Ralph et al., 2005; Voss et al., 2006; Dzikowski et al., 2007). The presence of PfSET10 in a subcompartment of the perinucleus suggested it could be a regulator of the active *var* in the expression site. We used

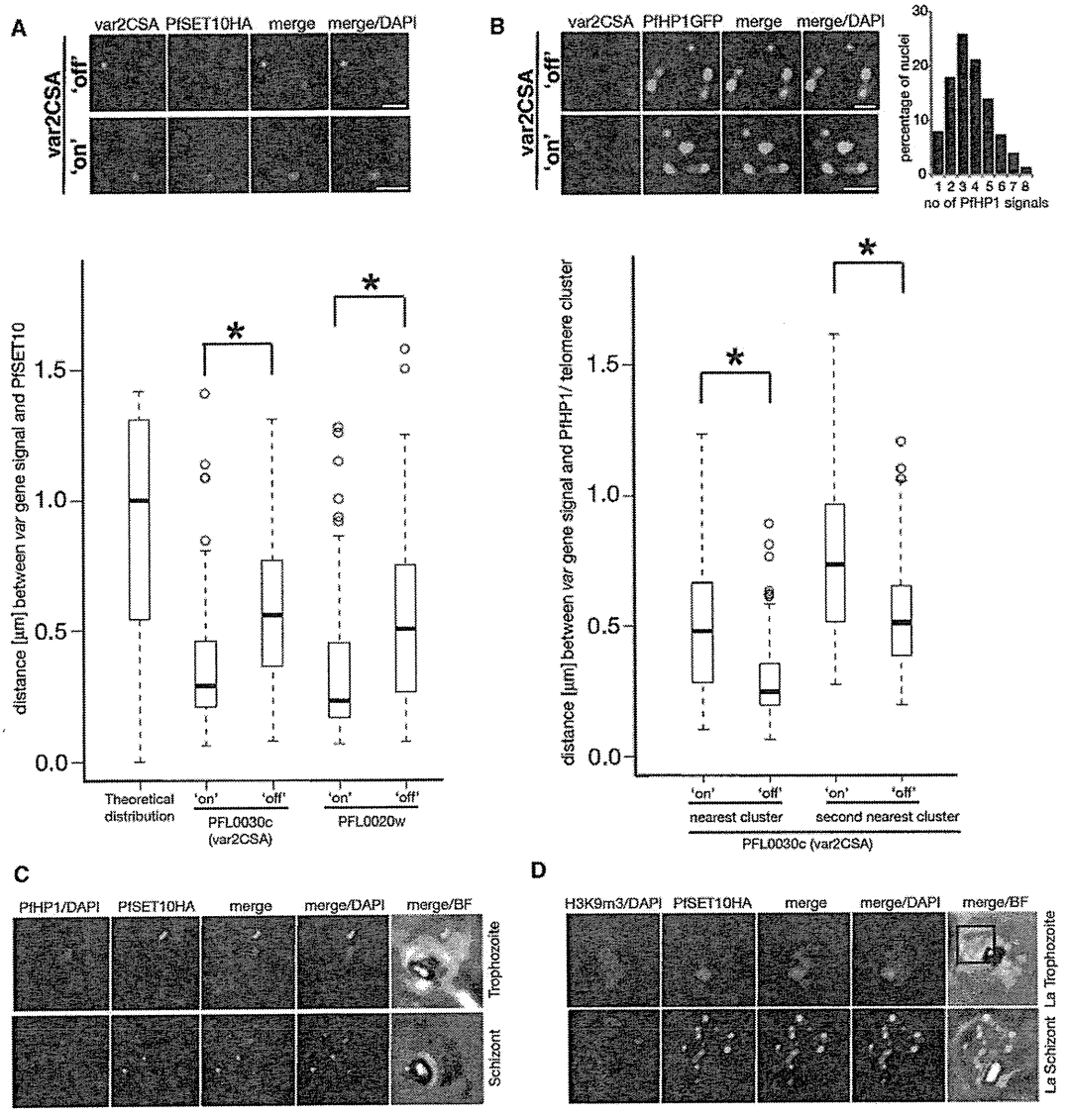

Figure 2. PfSET10 Colocalizes to the Activated *var* Gene

(A) IFA-FISH of 3D7SET10-HA, in which *var2csa* (PFL0030c) activation has been selected by CSA adherence ("on") (bottom panel). Unselected 3D7SET10-HA (top panel) has *var2csa* "off." The localization of *var2csa* (green) and PfSET10 (red) is shown with both merged with the nucleus stained by DAPI (blue).Scale bars are equivalent to 0.5 µm. Independently, another *var* gene (PFL0020w) has been selected for activation by ICAM adherence ("on"), and sample distributions (PFL0030c "on" n = 200; "off" n = 173; PFL0020w "on" n = 122; "off" n = 111) are plotted using a standard Tukey boxplot; * stands for $p < 0.000$.

(B) PfHP1 does not localize with active *var2csa* ("on"). The *var2csa* gene (red) location is shown with respect to PfHP1 protein (green). Right panel shows the distribution of PfHP1 signal numbers detected in 150 nuclei. Sample distributions ("on" n = 150; "off" n = 137) are plotted below using a standard Tukey boxplot; * stands for $p < 0.000$.

(C and D) (C) PfHP1 (red) and (D) histone mark H3K9me3 (red,), known markers of silent *var* gene clusters and PfSET10 (green), do not colocalize in the nucleus stained with DAPI (blue). See also Figure S2.

IFA-fluorescent in situ hybridization (IFA-FISH) (Flueck et al., 2009) to determine if PfSET10 colocalized with the active/ poised *var* gene (Figure 2). 3D7SET10-HA parasites were selected for expression of two *var* genes, *var2CSA* (PFL0030c) or PFL0020w ("on"), the genes encoding a *Pf*EMP1 specifying adherence to chondroitin sulfate A (CSA) (Salanti et al., 2003) and *i*ntercellular *a*dhesion *m*olecule-1 (ICAM) (Petter et al., 2011) respectively. As control, we used unselected 3D7SET10-HA ("off") parasites, in most of which *var2CSA* and PFL0020w were expected to be silent ("off"). Predominant *var2csa* and PFL0020w transcription in 3D7SET10-HA/CSA was confirmed by qRT-PCR (Figure S2A). The analysis was performed in late ring/early trophozoites stages, in which the previously active *var* gene enters the poised state, when PfSET10 was expressed