Targets of antibodies against *Plasmodium falciparum*–infected erythrocytes in malaria immunity

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***Plasmodium falciparum* is the major cause of malaria globally and is transmitted by mosquitoes. During parasitic development, *P. falciparum*–infected erythrocytes (*P. falciparum*–IEs) express multiple polymorphic proteins known as variant surface antigens (VSAs), including the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). VSA-specific antibodies are associated with protection from symptomatic and severe malaria. However, the importance of the different VSA targets of immunity to malaria remains unclear, which has impeded an understanding of malaria immunity and vaccine development. In this study, we developed assays using transgenic *P. falciparum* with modified PfEMP1 expression to quantify serum antibodies to VSAs among individuals exposed to malaria. We found that the majority of the human antibody response to the IE targets PfEMP1. Furthermore, our longitudinal studies showed that individuals with PfEMP1-specific antibodies had a significantly reduced risk of developing symptomatic malaria, whereas antibodies to other surface antigens were not associated with protective immunity. Using assays that measure antibody-mediated phagocytosis of IEs, an important mechanism in parasite clearance, we identified PfEMP1 as the major target of these functional antibodies. Taken together, these data demonstrate that PfEMP1 is a key target of humoral immunity. These findings advance our understanding of the targets and mediators of human immunity to malaria and have major implications for malaria vaccine development.**

Introduction

Malaria caused by the protozoan parasite *Plasmodium falciparum* is a major burden of disease globally, causing an estimated 225 million illness episodes and around 800,000 deaths per year (1). Young children are at highest risk of developing malaria, with *P. falciparum* being a leading cause of mortality among children under 5 years (2). There is an ongoing and urgent need for effective vaccines to advance the control and elimination of malaria, particularly in light of increasing drug resistance, including signs of emerging resistance to the artemisinin class of antimalarials (3), and concerning reports of the declining efficacy of vector control interventions in some regions (4).

The symptoms and clinical complications of malaria are caused by the erythrocytic stage of infection, and the majority of the acquired immune response is against these blood-stage parasites (5–7). The capacity for immune evasion enables *P. falciparum* to cause repeated and chronic infections; after repeated exposure to malaria, individuals eventually develop effective immunity that controls parasitemia and prevents severe and life-threatening complications (reviewed in ref. 8). Antibodies are an important component of acquired protective immunity (reviewed in ref. 9), and the passive transfer of immunoglobulin from immune donors to individuals with *P. falciparum* infection has been shown to reduce parasitemia and clinical symptoms (10).

During intraerythrocytic development, *P. falciparum* expresses antigens on the erythrocyte surface (11, 12). These antigens on infected erythrocytes (IEs) appear to be highly polymorphic and undergo clonal antigenic variation, and acquired antibodies against these antigens typically demonstrate a high degree of strain specificity (12, 13). Antigenic diversity and variation of surface antigens facilitates the development of repeated infections over time, as new infections appear to exploit gaps in the repertoire of variant-specific antibodies (5, 12). Prospective studies in children provide strong evidence that surface antigens are targets of protective immunity by showing that antibodies are associated with a reduced risk of developing malaria, and studies suggest that increasing exposure leads to a broad repertoire of antibodies that provides protection against different variants (5, 14–16). Antibodies to these IE surface antigens are thought to confer protection by inhibiting vascular adhesion and sequestration of IEs (17, 18) and by opsonizing IEs for phagocytic clearance (19).

Parasite-derived IE variant surface antigens (VSAs) include *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (11), repetitive interspersed family (RIFIN) proteins (20–22), subtelomeric variable open reading frame (STEVOR) proteins (23), surface-associated interspersed gene family (SURFIN) proteins (24), and possibly others (25). Modified host proteins such as erythrocyte band 3 have also

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been implicated as immune targets (26, 27). The most extensively studied VSA is PfEMP1, which is a major virulence factor and has been shown to be targeted by naturally acquired antibodies (5, 11, 28). PfEMP1 mediates the formation of erythrocyte rosettes and the adhesion of IEs to the vascular endothelium, which enables the parasite to sequester in various organs, such as the brain and placenta, thus contributing to the pathogenesis of malaria disease (reviewed in ref. 25). PfEMP1 is encoded by the highly polymorphic *var* multigene family (~60 genes per genome) (29–31), and the expression of PfEMP1 is clonally variant; different *var* genes encode PfEMP1 variants with different antigenic and adhesive properties (13, 30, 32). Through exclusive transcription, only one PfEMP1 variant is generally expressed on the IE surface at any time (33, 34). RIFIN, STEVOR, and SURFIN proteins are also encoded by polymorphic multigene families, but their functions and roles in acquired immunity are currently unclear. Recent data suggests that RIFIN and STEVOR proteins could be important antibody targets (35–38).

Dissecting which of the VSAs are antigenically dominant and targets of protective antibodies is crucial for understanding the relevance and importance of different VSAs as vaccine candidates and their role in immunity and host-parasite interactions. Until now, it has been difficult to directly quantify the importance of PfEMP1 as a target of acquired immunity to IE surface antigens, measure antibodies to native PfEMP1, or understand the significance of other surface antigens. Although studies have examined antibodies to recombinant domains from PfEMP1 or other VSAs like RIFIN (37), issues regarding the correct folding of recombinant proteins, the significance of the tertiary and/or quaternary structure of PfEMP1, and the selection of relevant domains to study have limited these approaches. The interpretation of data showing associations between antibodies to VSAs and protective immunity to malaria is greatly hampered by the lack of knowledge regarding the targets of these antibodies.

In this study, we developed an approach that we believe to be novel to quantify the importance of PfEMP1 and other VSAs as targets of protective antibodies, using *P. falciparum* isolates that were genetically modified to suppress PfEMP1 expression. *P. falciparum*–IEs were transfected with a construct that encodes a *var* promoter but lacks a downstream *var* gene. Under drug selection, the *var* promoter is expressed, which silences the endogenous *var* promoters and therefore inhibits PfEMP1 expression (39, 40). We applied these tools to human studies to quantify the importance of PfEMP1 and other surface antigens as targets of acquired antibodies, including antibodies that mediate opsonic phagocytosis of IEs, and identify responses linked with protective immunity among residents of a malaria-endemic area in Kenya.

Results

Generation and characterization of parasites with altered expression of PfEMP1. In this study, we used 2 *P. falciparum* isolates that had substantially reduced PfEMP1 expression to study antibody responses. Isolate 3D7vpkd (*var* promoter knockdown) was generated from 3D7 by transfection with a vector containing the *UpsC var* gene promoter and cultured under drug selection to suppress endogenous *var* gene expression, as previously described (40). The phenotype of 3D7vpkd parasites has been reported previously (40); here, we confirmed that 3D7vpkd reduced *var* gene expression in Northern blots (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI62182DS1) and significantly reduced adhesion to the CD36 receptor (Figure 1E). In this study, we generated the additional line, E8Bvpkd. E8B–ICAM-1 parasites were transfected with a vector containing the *UpsC* promoter and human DHFR, which encodes resistance to the antimalarial compound WR99210. Parasites were cultured with WR99210 to generate E8Bvpkd. Several approaches were taken to confirm the loss of PfEMP1 expression in this transfected line. Western blots showed markedly reduced expression of PfEMP1 when probed with a monoclonal antibody against the conserved C-terminal acidic terminal sequence of PfEMP1, compared with that of E8B parental parasites (Figure 1B). Adhesion of E8Bvpkd to ICAM-1 and CD36 receptors was also significantly reduced compared with that of parental parasites, suggesting PfEMP1 expression was reduced (Figure 1, C and D); however, it was interesting that some adhesion to CD36 was retained. These findings confirmed that transfection had effectively inhibited PfEMP1 expression in 3D7vpkd and E8Bvpkd parasites. In contrast, expression of another candidate surface antigen, RIFIN, appeared unchanged. Anti-RIF29 antibodies (37) labeled a protein of expected size in Western blots of IE membrane extracts (Figure 1B), and anti-RIF40 antibodies (37) labeled the erythrocyte membrane by indirect immunofluorescence assay (IFA) of mature trophozoite-IEs (Figure 2A); there was no labeling of uninfected erythrocytes with anti-RIF40 antibodies (Supplemental Figure 1D). Expression of the candidate surface antigen STEVOR was also detected in both parasite isolates. Anti-STEVOR10 antibodies (41) labeled the erythrocyte membrane of mature trophozoite 3D7 parental–IEs and 3D7vpkd-IEs by IFA (Figure 2B) as well as E8B parental–IE and E8Bvpkd-IEs (Supplemental Figure 1C); there was no labeling of uninfected erythrocytes with anti-STEVOR10 antibodies (Supplemental Figure 1E). IFAs confirmed that another IE membrane protein, *P. falciparum* erythrocyte membrane 3 (PfEMP3), remained expressed in 3D7vpkd parasites (Figure 2C). In addition, transmission electron microscopy of IEs demonstrated that mature trophozoite-IEs of 3D7vpkd parasites had erythrocyte membrane protrusions known as knobs (Figure 2D), as seen for 3D7 parental IEs, further suggesting that the expression and assembly of erythrocyte membrane proteins (other than PfEMP1) occurred normally in the vpkd parasites.

PfEMP1 is the major target of antibodies to the surface of parasitized erythrocytes. To quantify the importance of PfEMP1 and other VSAs as targets of human antibodies, we tested a selection of sera (n = 26) from adults exposed to malaria, residing in Kilifi, Kenya, for antibodies to IEs of 3D7 parental and 3D7vpkd parasites by flow cytometry. The overall IgG binding to the surface of erythrocytes infected with 3D7vpkd parasites was dramatically reduced compared with that of erythrocytes infected with 3D7 parental parasites (Figure 3A; $P < 0.0001$, median MFI levels of IgG binding to 3D7 parental parasites were 12.6 versus 3.9 for 3D7vpkd parasites). Sera from most individuals showed a marked reduction in IgG binding to 3D7vpkd parasites compared with 3D7 parental parasites (Figure 3B). All of the 26 adult serum samples that we tested were classified as positive for IgG binding to 3D7 parental parasites (antibody positivity is defined as IgG levels > mean + 3 SD of non-exposed controls); although 24 out of 26 samples were still considered positive for IgG binding to 3D7vpkd parasites, the magnitude of IgG reactivity was greatly reduced. These initial studies highlighted the value of these comparative assays using transgenic parasites and indicate that the majority of the antibody response observed in these samples can be attributed to PfEMP1, reflected in the difference between the IgG binding

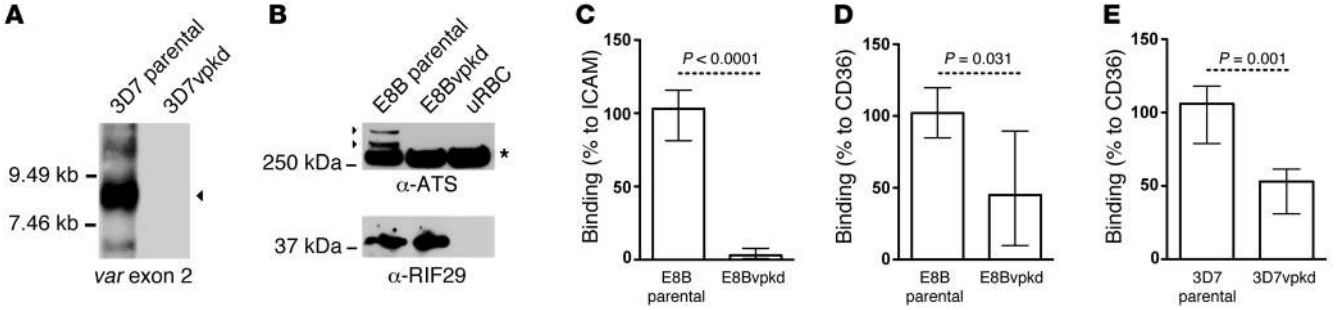

Figure 1

Phenotypic analyses of *var* promoter knockdown parasites. (**A**) Northern blot of *var* gene transcription by hybridization with a specific *var* exon 2 sequence. Compared with that in 3D7 parental parasites, *var* transcripts (arrow) are markedly reduced or absent in 3D7vpkd parasites. RNA was extracted from highly synchronous ring-stage IEs at approximately 10 hours after invasion. The position of molecular weight standards (kb) is indicated on the left. Ethidium bromide–stained gel prior to blotting was used as the loading control (Supplemental Figure 1B). (**B**) Western blot analyses of membrane extracts from mature trophozoite-IEs probed with anti-PfEMP1 and anti-RIF29 antibodies. In E8B parental parasites, full-length PfEMP1, which was absent in E8Bvpkd parasites, was detected at approximately 300 kDa (arrows). The double band represents different PfEMP1 variants expressed by E8B parental parasites. This anti-PfEMP1 antibody cross-reacts with erythrocyte spectrin (asterisk), as shown by comparison with extracts from uninfected erythrocytes (uRBC). The anti-RIF29 antibodies detected a protein at approximately 40 kDa, representing RIFIN in both E8B parental and E8Bvpkd parasites (bottom). ATS, acidic terminal sequence of PfEMP1. Adhesion of IEs to immobilized (**C**) ICAM-1 and (**D**) CD36 was significantly reduced in E8Bvpkd parasites compared with that in E8B parental parasites. (**E**) Adhesion of IEs to immobilized CD36 was significantly reduced in 3D7vpkd parasites compared with that in 3D7 parental parasites. However, adhesion to CD36 was partially retained in E8Bvpkd and 3D7vpkd parasites. Values are expressed as a percentage of parental parasites binding to each receptor. Assays were performed twice independently; bars represent median and interquartile ranges of samples tested in triplicate.

to 3D7 parental and 3D7vpkd parasites (3D7 parental minus 3D7vpkd). Our standard assays were performed testing human serum samples at a 1:10 dilution; when sera were tested at higher concentrations, the same pattern of reactivity to 3D7 parental and 3D7vpkd parasites was seen (Supplemental Figure 2, A and B). Additionally, we tested sera for reactivity to surface antigens using IEs at the schizont stage of intraerythrocytic development to account for possible differences in the relative expression of surface antigens at a later stage of parasite development, and studies have reported STEVOR expression on schizont-stage IEs (35). Results were very similar to those obtained using mature trophozoite-stage IEs, showing a marked reduction of IgG binding to 3D7vpkd parasites compared with that to 3D7 parental parasites (Supplemental Figure 2, C and D).

To confirm these findings in a genetically different parasite isolate, we tested the same selection of sera from adults from Kilifi for antibodies to E8B parental parasites compared with those to E8Bvpkd parasites (Figure 3, C and D). As seen for 3D7, the overall IgG binding to E8Bvpkd parasites by serum samples was greatly reduced compared with that to the parental parasites (Figure 3C; $P < 0.0001$, the median level of IgG binding for E8B parental parasites was 24.6 versus 9.2 for E8Bvpkd parasites), and PfEMP1 appeared to be the major target of antibodies for all sera tested (Figure 3D). There was a significant correlation between IgG binding to 3D7 parental and 3D7vpkd parasites (Spearman's rho [r_s] = 0.92, $P < 0.0001$) and between E8B parental and E8Bvpkd parasites ($r_s = 0.88$, $P < 0.0001$) (Supplemental Figure 3, A and B), suggesting that antibodies to PfEMP1 and other VSAs are co-acquired to some extent following *P. falciparum* infection. The correlation between IgG binding to E8B parental parasites and 3D7 parental parasites was also significant ($r_s = 0.64$, $P = 0.03$); however, the correlation between E8Bvpkd parasites and 3D7vpkd parasites was not significant ($r_s = 0.37$, $P = 0.17$) (Supplemental Figure 3, C and D), suggesting that there are substantial antigenic differences between non-PfEMP1 VSAs of different isolates and that these differences may be greater than those for PfEMP1. In order to validate our findings with the sera from adults from Kilifi, we also tested a selection of samples ($n = 36$) from children and adults in a neighboring community (Ngerenya), which showed a similar pattern of antibody recognition as described above, with substantially reduced IgG binding to 3D7vpkd parasites compared with that to 3D7 parental parasites (data not shown).

In order to expand these findings and obtain data on the acquisition of antibodies in a larger study, we tested serum samples from a cohort of 279 children (aged 6 months to 15 years), and a small number of adults for comparison ($n = 17$), residing in the Chonyi township, Kilifi, who had longitudinal follow-up for malaria episodes. As seen with other samples, the level of IgG binding to 3D7vpkd parasites was greatly reduced compared with that to 3D7 parental parasites (Figure 4A; $P < 0.0001$). Most sera showed a major reduction in IgG binding to 3D7vpkd parasites compared with that to 3D7 parental IEs (Figure 4B); 171 out of 296 samples were positive for IgG binding to 3D7 parental parasites, whereas only 56 out of 296 samples were positive for IgG binding to 3D7vpkd parasites. Of the 171 sera classified as positive to 3D7 parental IEs, 157 (92%) showed a reduction in IgG binding to 3D7vpkd of more than 80% compared with that of 3D7 parental IEs. In a further 9 samples (5%), IgG binding was reduced by 60% to 80% in 3D7vpkd, and in 5 samples (3%), it was reduced by less than 60%. Therefore, PfEMP1 appeared to be the major target of anti-VSA antibodies in 97% of sera with detectable antibodies to 3D7 parental parasites. This dominance of PfEMP1 antibodies was particularly evident in sera that had a high response to 3D7 parental parasites (Supplemental Figure 4A). There were insufficient sample volumes available to test responses to E8B isolates, in addition to 3D7, in this cohort. However, as 3D7 and E8B showed similar patterns of decreased reactivity following inhibition of PfEMP1 expression with our other sample

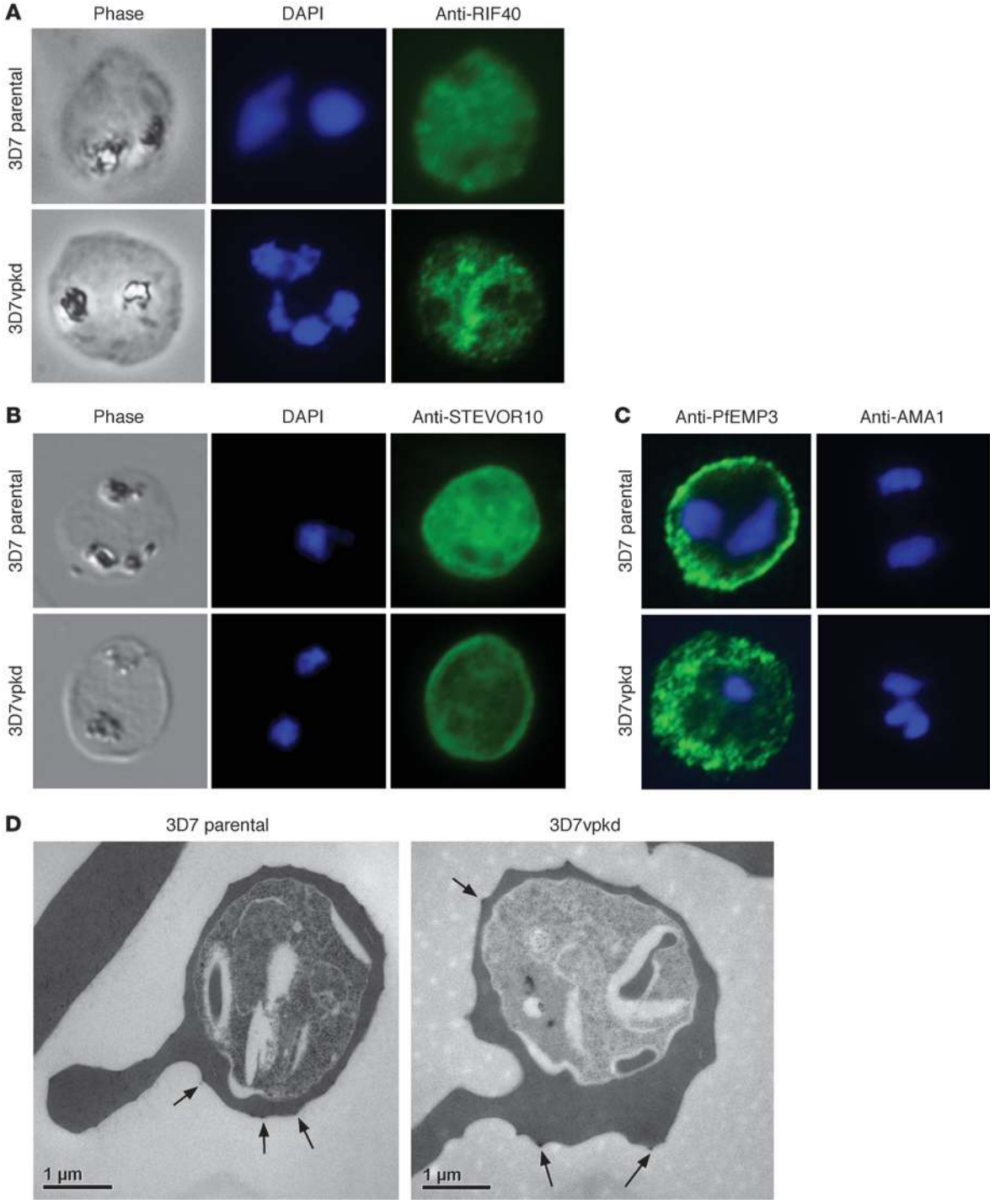

Figure 2
Exported proteins remained expressed by 3D7 parental and 3D7vpkd transgenic parasites. Immunofluorescence assays demonstrate the expression of (**A**) RIFIN and (**B**) STEVOR proteins by mature trophozoite-stage parasites (green). Despite the lack of PfEMP1 expression, RIFIN and STEVOR proteins were detectable in the transfected 3D7vpkd parasites similar to 3D7 parental parasites. (**C**) Mid-trophozoite-stage parasites from 3D7 parental and 3D7vpkd lines were probed with anti-PfEMP3 antibodies as a positive control and anti-AMA1 antibodies as a negative control (green). As expected, the pattern of staining by anti-PfEMP3 antibodies was consistent with labeling of PfEMP3 in the IE membrane, and there was no apparent labeling of AMA1. In all assays, cells were fixed with a mixture of acetone (90%) and methanol (10%), and DAPI was used to stain nuclear DNA (blue). (**A**–**C**) All images were taken with equal exposure for both parasite lines (original magnification, ×1000). (**D**) Electron-dense knobs in the erythrocyte membrane (arrows) were observed for IEs of 3D7 parental and 3D7vpkd parasites by transmission electron microscopy. Scale bar: 1 μm.

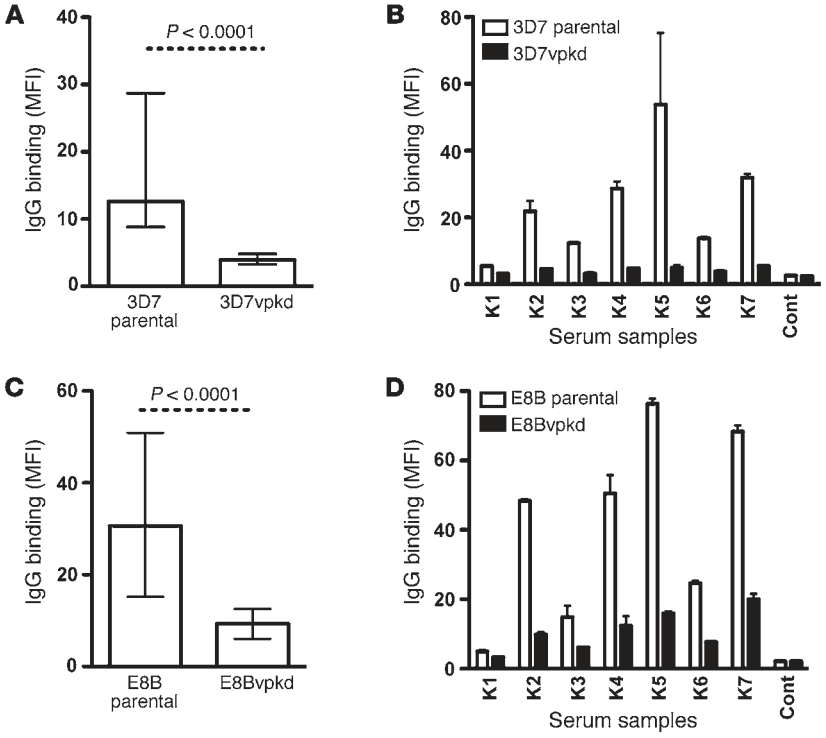

Figure 3
Antibodies among sera from Kenyan adults to surface antigens expressed by *P. falciparum*–IEs. (**A** and **C**) IgG binding to the surface of erythrocytes infected with 3D7vpkd and E8Bvpkd parasites was significantly reduced compared with that to (**A**) 3D7 parental and (**C**) E8B parental parasites. Assays were performed twice independently; bars represent median and interquartile ranges of samples tested in duplicate (n = 26 for 3D7; n = 22 for E8B). P values were calculated using a paired Wilcoxon signed-rank test. (**B** and **D**) A representative selection of serum samples tested for (**B**) antibodies to 3D7 parental and 3D7vpkd parasites and (**D**) antibodies to E8B parental and E8Bvpkd parasites. Samples tested were from adults (K1–K7) exposed to malaria residing in the Kilifi district, Kenya, and nonexposed Melbourne residents (Cont). IgG binding to 3D7vpkd and E8Bvpkd parasites was substantially reduced in most individuals. There was minimal reactivity observed among sera from Melbourne residents. Assays were performed twice independently; bars represent mean and range of samples tested in duplicate. IgG binding levels are expressed as geometric MFI for all graphs.

sets (Figure 3), we believe that the comparisons between 3D7 parental and 3D7vpkd parasites used in this cohort give a representative measure of responses to PfEMP1 and other antigens.

To complement these findings, sera that were high responders for IgG binding by flow cytometry were tested for their ability to agglutinate IEs, which reflects antibodies to surface antigens (12, 42). Samples selected for these assays included 10 that had high IgG binding to 3D7 parental and little response to 3D7vpkd parasites, and 10 that were reactive to both 3D7 parental and 3D7vpkd parasites. Of the 20 samples, 19 agglutinated 3D7 parental IEs substantially more than negative controls; each of these showed markedly lower agglutination of 3D7vpkd compared with that of 3D7 parental (Figure 4D; P < 0.0001 compared with 3D7 parental). Only 5 sera agglutinated 3D7vpkd parasites to a greater extent than nonexposed controls.

The targets of antibodies reactive with 3D7vpkd parasites were further investigated by evaluating the effect of trypsin treatment of IEs on antibody binding, as PfEMP1 is known to be highly sensitive to trypsin (11). Sera positive for IgG binding to 3D7vpkd parasites (n = 22) in the above experiments were tested in parallel against 3D7 parental and 3D7vpkd parasites, with or without trypsin treatment (10 μg/ml for 30 minutes) (Supplemental Figure 4, B and C). All sera showed a substantial reduction in IgG binding to 3D7 parental parasites after trypsin treatment, consistent with antibodies targeting the highly trypsin-sensitive PfEMP1; 17 of these 22 sera also had reduced binding to 3D7vpkd parasites after trypsin treatment. The overall reduction in IgG binding after trypsin treatment was greater for 3D7 parental than 3D7vpkd parasites (median reduction in reactivity was 82.8% versus 36.6%, respectively). Three of the five samples that did not have a reduction in reactivity after trypsin treatment at 10 μg/ml also did not show a reduction after treatment at 100 μg/ml, suggesting that antibodies predominantly target trypsin-sensitive antigens expressed by 3D7vpkd parasites, similar to that observed with parental parasites, but some antibodies may target trypsin-resistant antigens or epitopes on the surface of 3D7vpkd-IEs. However, the low level of antibody reactivity to 3D7vpkd parasites warrants caution in interpretation of these results. Similar assays with E8B parasites (n = 6 sera) revealed a reduction in antibody binding of 61.1% after trypsin treatment of E8B parental parasites compared with a reduction of 72.7% for E8Bvpkd parasites (Supplemental Figure 4, D and E).

PfEMP1-specific antibodies are associated with parasitemia and increasing age and exposure. There was a significant age-associated increase in IgG binding to IE surface antigens of both 3D7 parental (P < 0.0001) and 3D7vpkd parasites (P = 0.0001; Figure 4C), reflective of the acquisition of immunity in the population (43); however, the reactivity to 3D7vpkd-IEs was generally very low. The difference between IgG binding to 3D7 parental and 3D7vpkd parasites (3D7 parental minus 3D7vpkd) is interpreted as IgG specific to PfEMP1; this also increased with age (P = 0.0001 for comparison of different age groups). IgG binding to IEs was higher for those who were parasitemic at the time of sample collection compared with those who were not; this was seen for antibodies to 3D7 parental parasites (P < 0.0001) and for 3D7-PfEMP1–specific antibodies (P = 0.0001). However, IgG binding to 3D7vpkd parasites was not significantly higher among parasitemic individuals compared with that among aparasitemic individuals (P = 0.30; among parasitemic children median interquartile range [IQR] was 11.32[0–31.49] for 3D7 parental and 0[0] for 3D7vpkd parasites; among aparasitemic children median IQR was 0[0–14.27] for 3D7 parental and 0[0] for 3D7vpkd parasites).

Antibodies to PfEMP1 are associated with protection from symptomatic malaria. A key parameter for understanding the potential clinical significance of different antibody responses is their associa-

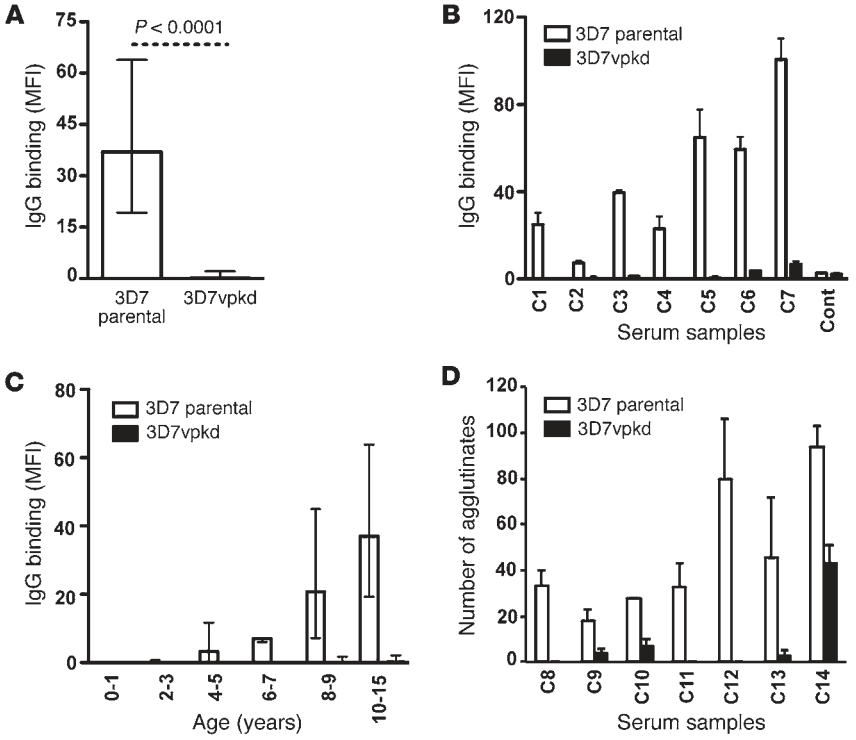

Figure 4

Antibodies among sera from Kenyan children and adults to surface antigens expressed by *P. falciparum*–IEs. (**A**) IgG binding to the surface of erythrocytes infected with 3D7vpkd parasites was markedly reduced compared with that of 3D7 parental parasites. Assays were performed twice independently; bars represent median and interquartile ranges of samples tested in duplicate (n = 296). The P value was calculated using a paired Wilcoxon signed-rank test. (**B**) A representative selection of samples tested for antibodies to 3D7 parental and 3D7vpkd parasites. Samples tested were from residents (C1–C7) exposed to malaria in the Chonyi cohort, Kenya, and nonexposed United Kingdom residents (Cont). IgG binding to 3D7vpkd parasites was substantially reduced in most individuals. There was minimal reactivity observed among sera from nonexposed United Kingdom residents. Assays were performed twice independently; bars represent mean and range of samples tested in duplicate. (**C**) Antibody responses among children of different age groups from the Chonyi cohort. Antibody acquisition was age-dependent as older children had higher levels of IgG binding to 3D7 parental parasites. Children from all age groups had very low IgG binding levels to 3D7vpkd parasites. Bars represent median and interquartile ranges. (**A**–**C**) IgG binding levels are expressed as geometric MFI for all graphs. (**D**) Antibodies to IE surface proteins measured by agglutination assays among a selection of sera from children (n = 20). A representative selection is shown (C8–C14); most individuals have antibodies that agglutinated 3D7 parental parasites to a much greater extent than 3D7vpkd parasites. Bars represent mean and range of samples tested in duplicate.

tion with protective immunity. To address this, we prospectively investigated the association between antibodies and risk of first episode of symptomatic malaria in the cohort; only children 1–10 years of age (n = 227) were included, since this is the group acquiring immunity (44), and excluding adults helped reduce the potential confounding effect of age. Analysis focused on children who were parasite positive at baseline (n = 112), as this is the group at risk of developing malaria in this cohort (43, 45). Our analysis revealed that children who were positive for IgG binding to 3D7 parental parasites (Figure 5A; hazard ratio [HR] = 0.23 [0.11–0.44], P < 0.001) and PfEMP1-specific IgG (defined as IgG binding to 3D7 parental minus 3D7vpkd; Figure 5C; HR = 0.24 [0.12–0.46], P < 0.001) had significantly reduced risk of developing clinical malaria. In contrast, the association between antibodies to 3D7vpkd parasites and risk of developing malaria was not significant (Figure 5B; HR = 0.61 [0.24–1.57], P = 0.31). After adjusting for the confounding effects of age, a reduced risk of developing malaria was still seen for those with antibodies to 3D7 parental or 3D7-PfEMP1 (adjusted HR = 0.47 [0.21–1.03], P = 0.059 for 3D7 parental; adjusted HR = 0.49 [0.22–1.08], P = 0.077 for 3D7-PfEMP1). These findings suggest that PfEMP1, rather than non-PfEMP1 antigens, is the key component of antibodies to the surface of IEs that are associated with protective immunity. We also assessed associations with malaria outcomes among children who were parasite negative at enrollment. However, the lower incidence of malaria in this group of children (43, 45) reduced statistical power to identify associations (odds ratio for the risk of malaria was 2.5 for parasitemic versus aparasitemic children). Univariate analysis showed no significant associations between malaria risk and antibodies to 3D7 parental, 3D7vpkd, or 3D7-PfEMP1 (HR = 1.45 [0.66–3.15], P = 0.350; HR = 0.51 [0.12–2.15], P = 0.358; HR = 1.49 [0.69–3.26], P = 0.309; respectively).

PfEMP1 is the major target of antibodies that mediate opsonic phagocytosis. To further address the relevance of these findings, we assessed the functional activity of these antibodies by testing them for the ability to opsonize IEs for phagocytosis by undifferentiated THP-1 monocytes using the different parasite lines, 3D7 and E8B (Figure 6). Undifferentiated THP-1 monocytes were used because they have very little nonopsonic phagocytic activity and therefore are valuable for measuring opsonic activity of antibodies (46). The ability of serum antibodies to promote opsonic phagocytosis activity was significantly reduced with the 3D7vpkd parasites compared with that with 3D7 parental parasites (Figure 6A; P < 0.0001, median phagocytic index was 16.3% for 3D7 parental and 3.6% for 3D7vpkd). As seen for 3D7, the opsonic phagocytosis activity was also significantly reduced with the E8Bvpkd parasites compared with that with the E8B parental parasites (Figure 6C; P < 0.0001, median phagocytic index was 32.2% for E8B parental and 11.7% for E8Bvpkd). All sera that we tested showed reduced opsonic phagocytosis of both 3D7vpkd and E8Bvpkd parasites compared with that of their parental parasites (Figure 6, B and D), suggesting that PfEMP1-specific antibodies are required to opsonize IEs for efficient clearance by phagocytes.

The correlation between opsonic phagocytosis activity of 3D7 parental parasites and 3D7vpkd parasites was highly significant (r_s = 0.80, P < 0.0001) as was the correlation between opsonic phagocytosis activity of E8B parental parasites and E8Bvpkd parasites (r_s = 0.82, P < 0.0001) (Supplemental Figure 5, A and B). The correlation between opsonic phagocytosis activity of E8B paren-

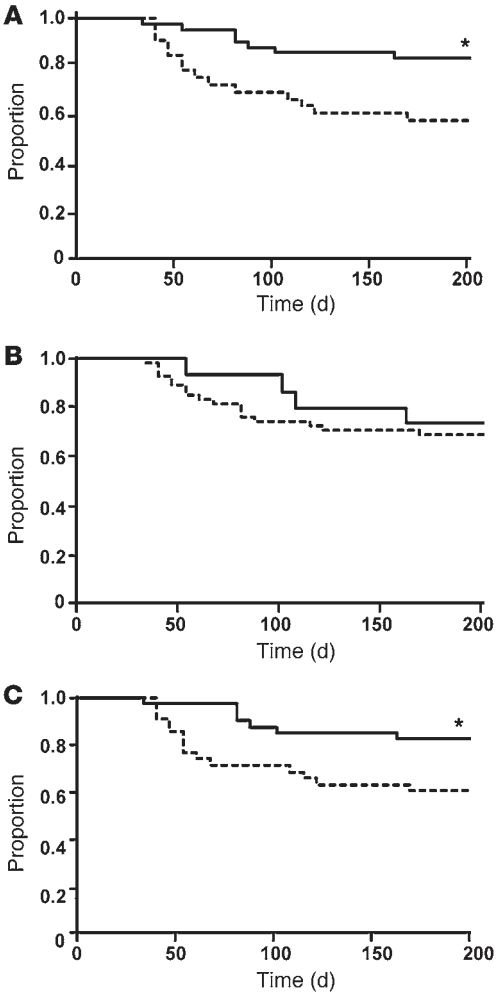

Figure 5
Antibodies to IEs and the risk of symptomatic *P. falciparum* episode during follow-up. Kaplan-Meier survival curves show the proportion of children who remained free of malaria episodes over time for IgG responses to (**A**) surface antigens of 3D7 parental parasites, (**B**) surface antigens of 3D7vpkd parasites, and (**C**) 3D7-PfEMP1 (defined as IgG binding to 3D7 parental minus 3D7vpkd). Solid lines represent children who are positive for IgG binding; dashed lines represent children who are negative for IgG binding. **P* < 0.05, comparing antibody-positive and antibody-negative individuals (log-rank test). Unadjusted data are shown. Symptomatic *P. falciparum* infection was defined as fever plus a parasite load of 2,500 parasites per μl. Survival analysis was restricted to children between 1 and 10 years of age who were parasite positive at baseline (*n* = 112).

tal parasites and 3D7 parental parasites was highly significant (r_s = 0.82, *P* = 0.005), but the correlation between opsonic phagocytosis activity of E8Bvpkd parasites and 3D7vpkd parasites was not significant (r_s = 0.40, *P* = 0.15) (Supplemental Figure 5, C and D). Furthermore, IgG reactivity measured by flow cytometry to E8B parental, E8B-PfEMP1, and E8Bvpkd parasites was significantly correlated with opsonic phagocytic activity (Figure 6, E and F; r_s = 0.76, *P* < 0.0001 for E8B parental; r_s = 0.72, *P* < 0.0002 for E8B-PfEMP1; r_s = 0.57, *P* = 0.006 for E8Bvpkd). Furthermore, IgG reactivity to 3D7 parental and 3D7-PfEMP1 was also significantly correlated with opsonic phagocytic activity (r_s = 0.51, *P* = 0.004 for 3D7 parental; r_s = 0.46, *P* = 0.009 for 3D7-PfEMP1) but 3D7vpkd was not (r_s = 0.19, *P* = 0.18) (Supplemental Figure 5, E and F).

Discussion

Prior studies have provided substantial evidence supporting the importance of antibodies to IE surface antigens in protection against clinical malaria in humans (5, 14–16). However, the relative importance of each of the several candidate VSAs to the overall antibody response and protective immunity has not yet been determined, due in part to technical constraints and lack of tools to dissect specific responses. Using approaches with transgenic parasites, we have quantified the importance of PfEMP1, relative to other VSAs, as a target of acquired human antibodies. Our striking findings reveal, for what we believe to be the first time, that the majority of the antibody response targets PfEMP1 and that this holds true for 2 genetically different parasite lines, 3D7 and E8B. We also found that PfEMP1 was the major target of antibodies to IE surface antigens in the great majority of the samples that we tested that were positive for IgG binding to 3D7 parental or E8B parental IEs. In most of these samples, antibodies to PfEMP1 accounted for more than 80% of the antibody binding, suggesting that it is the dominant VSA of *P. falciparum*–IEs.

The proposed importance of PfEMP1 in immunity was further emphasized by showing that it is the key target of antibodies to the surface of IEs that are associated with protective human immunity. Individuals with high levels of antibodies to 3D7 parental or 3D7-PfEMP1–specific antibodies had a significantly reduced risk of symptomatic malaria. In contrast, antibodies to 3D7vpkd showed no significant association with malaria risk, suggesting that non-PfEMP1 antigens are either not an important component of the protective response or that they play a minor role compared with that of antibodies to PfEMP1. The development of immunity to malaria in our study population occurs during childhood (43). The levels and prevalence of PfEMP1-specific and non-PfEMP1 antibodies showed a clear increase with age during childhood, consistent with the acquisition of immunity in this population. This finding also suggests that repeated infections over time are required to generate antibody responses toward both PfEMP1 and non-PfEMP1 surface antigens. Limiting our survival analysis to children aged 1–10 years helped reduce the potential confounding effects of age. After further adjusting the survival analysis for the age of children, a strong association between antibodies and reduced malaria risk remained (HR = 0.47 for 3D7 parental and HR = 0.49 for 3D7-PfEMP1), although the level of statistical significance was weakened. Higher antibody levels among individuals with parasitemia suggest antibody boosting occurred with infection or that this group had a higher level of exposure. A protective association between antibodies and malaria was only observed among children with active parasitemia at the time of enrollment when antibodies were measured. The lower incidence of malaria among children who were aparasitemic greatly reduced our statistical power to detect associations between antibodies and malaria in that group. It is possible that active parasitemia boosted antibody responses that contribute to protection. Further studies in other populations will be valuable to address these questions and understand their significance.

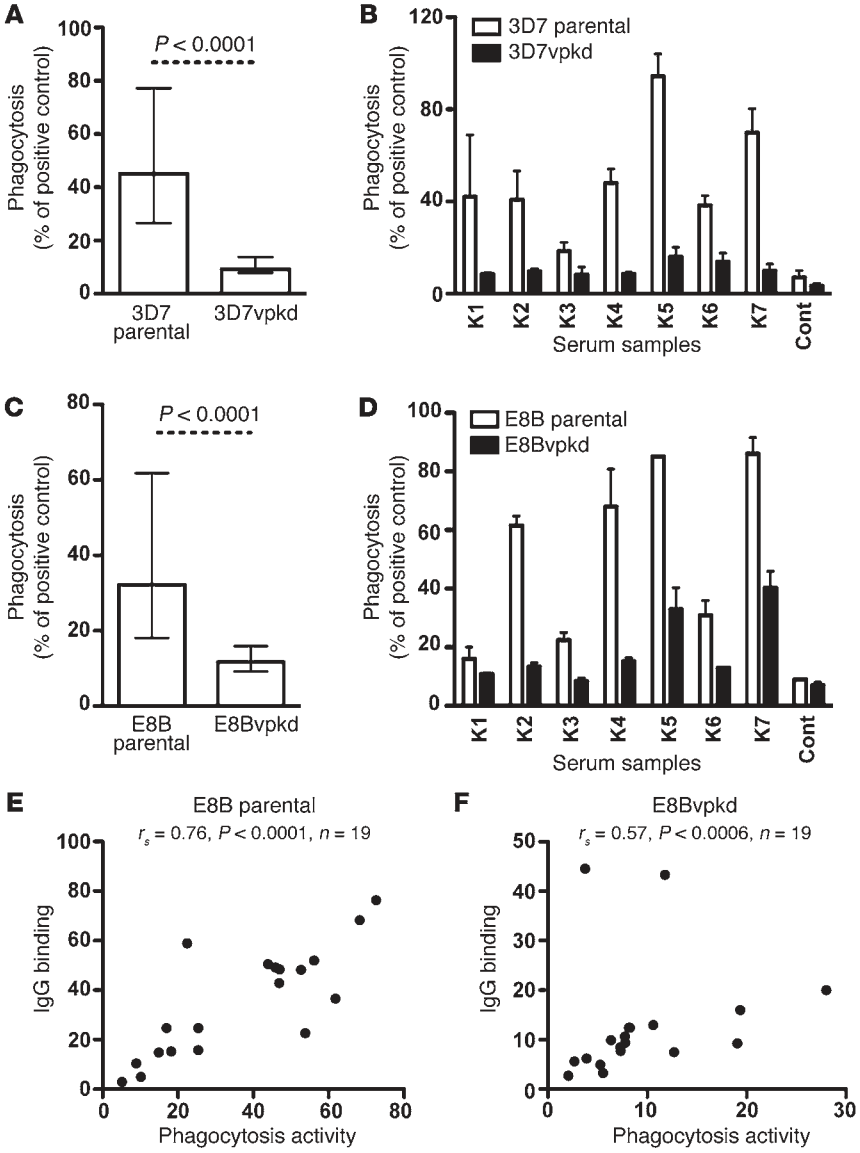

Figure 6
Opsonic phagocytosis of *P. falciparum*–IEs by undifferentiated THP-1 monocytes using sera from adults from Kilifi. (**A** and **C**) Opsonic phagocytosis activity of sera was significantly reduced in 3D7vpkd and E8Bvpkd parasites compared with that in (**A**) 3D7 parental and (**C**) E8B parental parasites. The level of phagocytosis is expressed as a percentage of the positive control for all graphs. Assays were performed twice independently; bars represent median and interquartile ranges (n = 24 for 3D7; n = 31 for E8B). P values were calculated using a paired Wilcoxon signed-rank test. (**B** and **D**) A representative selection of sera tested for phagocytosis activity with (**B**) 3D7 and (**D**) E8B parasites is shown. Samples were from malaria-exposed adults (K1–K7) residing in the Kilifi district, Kenya, and nonexposed Melbourne residents (Cont). In most samples, opsonic phagocytosis activity to 3D7vpkd and E8Bvpkd parasites was substantially reduced compared with that to (**B**) 3D7 parental and (**D**) E8B parental parasites. Assays were performed twice independently; bars represent mean and range, with samples tested in duplicate. The correlation between antibody levels measured as IgG binding and as opsonic phagocytosis activity is shown for (**E**) E8B parental and (**F**) E8Bvpkd parasites. Symbols represent results for individual serum samples.

If immunity to malaria is mediated by antibodies to PfEMP1, it may be that a broad repertoire of antibodies to different variants is required, given that PfEMP1 is a highly polymorphic protein; however, some data suggest that this antibody repertoire may not need to be extensive, as there may be a restricted number of common PfEMP1 variants that are important targets of immunity (47–49). Alternatively, protective antibodies may target cross-reactive, or even conserved, epitopes that give broad protection against the diversity of infecting variants (12). However, very little is currently known about the extent of antigenic diversity or relatedness between different PfEMP1 variants and the extent to which human antibodies target conserved and polymorphic epitopes. This is an important issue for future research. Our finding that antibodies to 3D7-PfEMP1 are associated with protective immunity in the study population may reflect the presence of antibodies that have broad cross-reactivity against different isolates or antibodies to PfEMP1 variants that are common in the study population and similar to those expressed by 3D7. Alternatively, antibodies to 3D7-PfEMP1 may be a marker of a broad repertoire of antibodies to different PfEMP1 variants that are present in protected children. Antibodies to IE surface antigens are thought to act in part by opsonizing IEs for clearance by monocytes and macrophages in the circulation and spleen (19). Importantly, we showed that antibody-mediated opsonic phagocytosis was significantly reduced in vpkd parasites of both 3D7 and E8B lines, suggesting that PfEMP1 is the major target of these functional antibodies. Together with findings on associations with protection, these results provide further evidence of an important role for PfEMP1 as a target of protective immunity. Interestingly, there was some measurable opsonic phagocytic activity with vpkd parasites. This suggests that the low level of IgG reactivity seen to the vpkd parasites, which may represent antibodies to non-PfEMP1 antigens, may still contribute to clearance of IEs and protective immunity.

The 3D7vpkd and E8Bvpkd lines used here were generated by transfecting parental parasites with a *var* promoter construct that lacked any coding sequence for PfEMP1 (40). Under drug selection, this approach substantially reduced endogenous PfEMP1 production, thus creating a PfEMP1-deficient line. This

was supported by the reduced or absent *var* gene expression by Northern blots, the lack of detectable PfEMP1 in Western blots of IE membrane extracts, and the markedly reduced adhesion of vpkd parasites to vascular receptors CD36 and ICAM-1. In our study, we demonstrated that other candidate surface proteins, RIFIN and STEVOR, were still expressed by these vpkd-transfected parasites, suggesting that protein trafficking and export was not affected. Furthermore, the expression and trafficking of PfEMP3 and knob-associated histidine-rich protein (KAHRP) (40) appeared to occur normally. The demonstration that the 3D7vpkd-IEs still express erythrocyte membrane knobs further suggests that the expression and assembly of membrane proteins, other than PfEMP1, occurs normally in the vpkd parasites. While it is possible that there could be disruption of other parasite-derived surface proteins if these normally exist in a complex with PfEMP1, at present, there are no published data to suggest that this is the case (50), and collectively our data suggest this is an unlikely explanation for our results.

The identity of the targets of antibodies to surface antigens expressed by 3D7vpkd and E8Bvpkd parasites is unclear. These antigens may include RIFIN, STEVOR, and SURFIN proteins, which have been identified on the IE surface (21, 22, 24, 35, 38). While it remains possible that some of the reactivity to vpkd parasites represents antibodies to residual PfEMP1 on the IE surface (if *var* gene expression is not completely inhibited in the vpkd lines), our Western blot analysis suggests that residual PfEMP1 is minimal. The proportion of the IgG response to 3D7vpkd compared with that to 3D7 parental IEs or to E8Bvpkd compared with that to E8B parental IEs varied between sera, therefore suggesting residual PfEMP1 is unlikely to account for all IgG binding to 3D7vpkd or E8Bvpkd parasites, and antibodies to other VSAs are likely explanations. Previous reports indicate that PfEMP1 is highly sensitive to cleavage by trypsin compared with other surface antigens, such as RIFINs that are partially trypsin resistant (11, 21). We found that IgG reactivity to 3D7 parental parasites was highly trypsin sensitive, consistent with PfEMP1 being the major target of antibodies to the IE surface of these parasites. IgG reactivity to 3D7vpkd was less sensitive to trypsin treatment, and some serum antibodies appeared to target trypsin-resistant epitopes expressed by 3D7vpkd. This finding suggests that non-PfEMP1 antigens may also be targets of human antibodies. Adhesion to ICAM-1 was almost completely absent in E8Bvpkd-IEs, whereas a significant level of adhesion to CD36 was retained in both E8Bvpkd and 3D7vpkd parasite lines, even though there was little or no detectable PfEMP1 or *var* expression. Currently, PfEMP1 is thought to be the sole parasite ligand for adhesion to ICAM-1 (50), and our findings are consistent with that. However, other ligands have been suggested for adhesion to CD36, such as parasite-modified erythrocyte Band 3 (26). It is possible that the residual CD36 adhesion may represent binding activity of other antigens, or alternatively a very low level of PfEMP1 expression may be sufficient to mediate adhesion to CD36. This issue warrants investigation in future studies.

Our findings represent a major advance in understanding VSAs as vaccine candidates by clearly establishing the importance of PfEMP1 relative to other candidate antigens and developing methods and approaches to more precisely measure antibodies to PfEMP1 and other VSAs that can be used in future immunity studies and vaccine trials. IE surface antigens have long been regarded as attractive vaccine candidates because of their importance as targets of acquired immunity, their key role in disease pathogenesis, and their prominent exposure to the immune system. However, one major roadblock to their development as vaccines has been a lack of understanding of the relative importance of the different candidate antigens, which we address here. A further barrier has been their level of antigenic diversity. In this respect, recent data on PfEMP1 suggest that diversity may not be as great as previously thought and that only a subset of variants may be responsible for causing severe malaria illness (47, 48, 51). If these variants are found to be antigenically restricted, the prospect of a multivalent vaccine based on PfEMP1 may be possible. Additionally, knowledge of key immune targets is valuable for developing serological approaches for malaria surveillance, identifying populations at risk, and evaluating the impact of malaria control interventions on malarial immunity. There is increasing interest in using serological assays as low-cost tools for surveillance of malaria exposure in populations to guide control efforts (52, 53). Our data showing the dominance and importance of PfEMP1 as an immune target suggest it would be a valuable antigen for use in serologic assays for malaria surveillance programs.

In conclusion, this study provides major evidence that antibodies to PfEMP1 are the most abundant and functionally important antibodies to VSAs on *P. falciparum*–IEs. Our findings suggest that PfEMP1 is a major target of antibodies that clear parasitemia and protect from clinical malaria. Furthermore, we have developed powerful approaches which we believe to be novel, which we refer to as transgenic parasite comparison assays, to measure PfEMP1-specific responses and dissect components of protective immunity. Such approaches illustrate the value of translating molecular approaches to clinical immunology. These findings, therefore, have significant implications for understanding and measuring immunity to malaria that are relevant for the development of malaria vaccines or approaches to monitor immunity and malaria exposure in populations.

Methods

Study population and ethics statement. Samples were obtained from a longitudinal cohort study of 296 individuals conducted in Chonyi, an area of high malaria transmission in Kilifi district, by the Centre for Geographic Medicine Research, Coast. The cohort has been extensively described elsewhere (43). In October 2000, each individual in the cohort had a serum sample taken, and a blood slide was read to determine their pre-surveillance infection status. Individuals were afebrile and asymptomatic at the time of sampling. They were then followed weekly for 210 days by active surveillance for fever or illness. Symptomatic malaria was defined as fever with an axillary temperature greater than 37.5°C, and *P. falciparum* parasitemia of greater than 2,500 parasites per μl for children above 1 year of age, and fever plus any parasitemia for infants. These have been determined to be sensitive and specific malaria case definitions in this community (43). Of the 296 individuals, most (n = 270) were aged between 6 months and 10 years (0–1 years, n = 43; 2–3 years, n = 56; 4–5 years, n = 60; 6–7 years, n = 56; 8–10 years, n = 55), and 26 were older than 10 years of age (11–15 years, n = 9; adults [aged 16–53], n = 17). This study focused largely on children aged 1–10 years, because this is the age range during which malaria immunity was acquired in this population (43). Samples were also tested from anonymous adult donors in the Kilifi district (n = 26) and from the Ngerenya cohort of children and adults (n = 36), which has been previously described (43). For negative controls, samples from malaria-naive residents of Melbourne, Australia, and the United Kingdom were used.

P. falciparum culture and isolates. *P. falciparum* isolates were maintained in continuous culture in RPMI-HEPES culture medium (Gibco) containing 10% pooled human serum (42, 54). Genetic identity of parasite isolates was confirmed by sequencing the *ama1* and *var2csa* genes (55). Parasites were synchronized using sorbitol (Sigma-Aldrich) treatment (56), and knob-expressing parasites were enriched by gelatin (Sigma-Aldrich) flotation (57). *P. falciparum* isolate E8Bvpkd was generated by transfecting the E8B-ICAM parental isolate (58), which is a clone of IT4, with the plasmid vector pHBupsCB^R (59) and culturing in the presence of WR99210 (2.5 nM) to inhibit endogenous *var* gene expression. The 3D7vpkd isolate was generated as previously described by transfecting parental 3D7 with a modified pHBupsC^R vector and culturing in the presence of blasticidin-S-HCl (Merck; 2.5 μg/ml) (40).

Measuring antibodies by flow cytometry. Testing for IgG binding to the surface of IEs by flow cytometry was performed as previously described (58) using mature trophozoite-IEs. Briefly, parasites at 0.2% hematocrit were consecutively incubated with test plasma or serum (1:10), polyclonal rabbit anti-human IgG (1:100, Dako), and Alexa Fluor 488–conjugated donkey anti-rabbit IgG (1:500, Invitrogen) with ethidium bromide (1:1,000, Bio-Rad), with washing between steps. All dilutions were performed in PBS with 0.1% casein (Sigma-Aldrich), and all incubations were at room temperature for 30 minutes. All samples were tested in duplicate. Sera from Melbourne residents were used as negative controls and samples from adults exposed to malaria were used as positive controls in the assays. Data was acquired by flow cytometry (FACSCalibur, BD Biosciences) and analyzed using FlowJo software. IgG binding levels for each sample were expressed as the geometric MFI (arbitrary units) for IEs, after subtracting the MFI of uninfected erythrocytes. Samples were designated antibody positive if the MFI was more than 3SD above the mean of reactivity seen with nonexposed control sera. IEs used in assays were either fresh from culture or cryopreserved and stored at –80°C and then thawed before use (60). Direct comparisons of fresh or cryopreserved parasites in antibodies assays revealed no significant differences in results (data not shown). For cryopreservation, cultures containing mature trophozoite-IEs were pelleted, and 0.2-times the pellet volume of glycerolyte was slowly added. After standing for 5 minutes, a further 2-times the pellet volume of glycerolyte (Baxter) was added. Samples were then stored at –80°C until required. Prior to use in assays, IEs were thawed and resuspended slowly in an equal volume of malaria thawing solution (MTS; 3.5% NaCl in distilled water) while agitating and allowed to stand for 2 minutes. Another 2 ml of MTS was added; cells were centrifuged at 300 *g* for 4 minutes, and the supernatant removed. The cell pellet was then resuspended in 2 ml of a 50:50 mix of MTS and PBS and centrifuged. This step was repeated using PBS only. Finally, cells were resuspended in the appropriate buffer for assays.

Agglutination assays. Sera were tested in agglutination assays using trophozoite-IEs (parasitemia 3.5%–4%) (42, 48). IEs (11.25 μl; hematocrit 5%) were incubated with 1.25 μl test sera (final concentration 1:10) in a 96-well plate on a rotating wheel for 1 hour at room temperature. Each sample was gently spread onto a glass slide with a micropipette tip over an approximately 15-mm diameter area and allowed to air dry. Smears were fixed with methanol and stained with 3% Giemsa for 30 minutes. Each sample was run in duplicate, and experiments were performed twice. Pooled sera from nonimmune United Kingdom donors were used as a negative control, and pooled Kenyan adult sera were used as a positive control. The whole slide was examined by light microscopy at ×10 magnification. The total number agglutinates (at least 5 IEs in size) were counted for each sample; no agglutinates were seen with negative control serum. Samples were coded and counted blindly.

Western blots. Western blots were performed using Triton X-100–insoluble, SDS-soluble protein extracts of trophozoite-IEs, as previously described (61, 62). Triton X-100–insoluble protein extracts were resuspended in 2% SDS in PBS and separated by SDS-PAGE performed on 3%–8% Tris-Acetate Gels (Invitrogen) for PfEMP1 detection and 4%–12% Bis-Tris Gels (Invitrogen) for RIFIN and STEVOR detection. Nitrocellulose membranes (Invitrogen) were probed with affinity-purified rabbit antiserum against the conserved acidic terminal sequence of PfEMP1 (1:1,000) (61) or affinity-purified rat antibodies against recombinant RIF29 protein (1:2,000; against a conserved region of RIFIN proteins) (accession no. AF483817) (37, 63). Protein extracts from uninfected erythrocytes were used as controls for antibody cross-reactivity.

Northern blots. TRIzol reagent (Invitrogen) was used to extract RNA from highly synchronous ring-stage parasite cultures at 10 hours after invasion. Northern blots were hybridized with a conserved *var* exon 2 sequence and washed at low stringency with 2X SSC and 0.1% SDS at 55°C, as previously described (64).

Immunofluorescence microscopy. Thin blood smears of trophozoite-IEs were fixed in 90% acetone and 10% methanol for 5 minutes at –20°C, as previously described (65). Briefly, slides were blocked in 1% BSA in PBS for 30 minutes and incubated with primary antibodies (anti-RIF40, 1:50; affinity-purified mouse antibodies against recombinant STEVOR protein (41); anti-STEVOR10, 1:200; anti-PfEMP3, 1:500; anti-AMA1, 1:500) followed by the corresponding Alexa Fluor 488–conjugated IgG (1:500). Slides were mounted in VectaShield (Vector Laboratories) with 0.1 ng/ml DAPI (Invitrogen) to label the parasite nucleus. All wash steps were performed with PBS, and incubations were conducted for 2 hours at room temperature. Images were obtained using a Plan-Apochromat (×100 oil, numerical aperture 1.40) oil immersion phase-contrast lens (Carl Zeiss) on an AxioVert 200M microscope (Carl Zeiss) equipped with an AxioCam Mrm camera (Carl Zeiss). Images were processed using Photoshop CS4 (Adobe).

Opsonic phagocytosis assays. Phagocytosis assays using human sera to opsonize the surface of trophozoite-IEs were conducted as previously described (46). Briefly, trophozoite-IEs were enriched by magnet purification (MACS, Miltenyi Biotec) to 95% parasitemia and incubated with ethidium bromide for 30 minutes in the dark. IEs were incubated with heat-inactivated serum samples for 1 hour at room temperature, followed by incubation with undifferentiated THP-1 cells (promonocytic cell line) for 40 minutes in a humidified incubator (37°C, 5% CO_2) to allow phagocytosis to occur. Phagocytosis was stopped by centrifugation at 4°C and 350 *g* for 3 minutes, and remaining nonphagocytosed IEs and uninfected erythrocytes were lysed for 10 minutes with FACS Lysing Solution (BD Biosciences). Cells were subsequently fixed in cold 2% paraformaldehyde in PBS, and the proportion of THP-1 cells that had phagocytosed IEs was counted by flow cytometry (FACSCalibur, BD Biosciences) for each sample. As described and validated previously (46), the level of phagocytosis for each sample was expressed relative to the positive control, which was rabbit antibody raised against human erythrocytes. Negative control samples (nonexposed Melbourne residents) were included in all assays.

P. falciparum adhesion assays. Adhesion assays were performed as previously described (42, 66) using gelatin-enriched *P. falciparum* trophozoite-IEs at 15%–20% parasitemia. Incubations were conducted at 37°C for 30 minutes, and wash steps were performed with plain RPMI-HEPES. Bound cells were fixed in 2% glutaraldehyde in PBS, stained with Giemsa, and counted by microscopy. Adhesion to each receptor was tested in triplicate with the indicated concentrations: ICAM-1, 10 μg/ml (Bender MedSystems), and CD36, 20 μg/ml (rhCD36/Fc Chimera, R&D Systems). *P* values were calculated using an unpaired *t* test.

Electron microscopy. Mature trophozoite-IEs were fixed in 1% glutaraldehyde and 1% Osmium for 30 minutes at 4°C, dehydrated, and embedded in LR White resin (ProSciTech). Ultrathin sections were cut using a Leica Ultracut microtome. Sections were stained after with uranyl acetate and lead citrate and analyzed using a Philips CM120 BioTwin Transmission Electron Microscope.

Statistics. Statistical analysis was performed using Microsoft Excel, STATA version 8, and Prism version 5 (GraphPad Software Inc.). *P* values for comparison between groups were determined using Mann-Whitney *U* tests and Wilcoxon signed-rank tests (2 tailed) for continuous variables or χ^2 test for proportions. A *P* value of less than 0.05 was considered statistically significant. Kaplan-Meier survival curves were generated for the time to first episode of clinical malaria, comparing children who were classified as IgG positive to 3D7 parental, 3D7vpkd, or PfEMP1-specific reactivity to those who are classified as IgG negative to these parasites; *P* values were determined using the log-rank test. Cox proportional hazards models were used to calculate HRs for the association of antibody variables with risk of symptomatic *P. falciparum* malaria (16); only first episodes were considered in the analysis. Analysis was performed until day 210, with the first 30 days excluded to reduce the effect of baseline parasitemia accounting for malaria episodes in the follow-up period. Survival analysis was restricted to children from 1 year to 10 years of age (n = 227), because prior studies have shown that associations between antimalarial antibodies and protective immunity are restricted to this group. This was also the age range during which malaria immunity was acquired in this population (43). Adults were excluded to reduce the potential confounding effect of age and because few adults experienced malaria during follow-up. For survival analysis, subjects were stratified by the presence or absence of peripheral blood *P. falciparum* parasitemia at the time of enrollment, because prior studies have shown an interaction among antibodies, parasitemia at baseline, and malaria outcomes in this cohort (45). Our analyses focused on the parasite-positive group because protective associations with antibodies are not observed among those who were parasite negative at sampling time as this group had a very low incidence of malaria (16, 44, 45). The correlation between antibody levels measured as IgG binding and opsonic phagocytosis activity, and between parasite lines, was determined using Spearman's rho (r_s).

Study approval. Ethics approval was obtained from the Kenya Medical Research Institute Ethics Committee, the Walter and Eliza Hall Institute Human Research and Ethics Committee, and the Alfred Hospital Human Research and Ethics Committee. Written informed consent was obtained from all study participants or their legal guardians.

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