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Erythrocyte-binding antigens of *Plasmodium falciparum* are targets of human inhibitory antibodies and function to evade naturally acquired immunity

Kristina E. M. Persson[*,†], **Freya J. I. Fowkes**[†,‡], **Fiona J. McCallum**[†], **Nimmo Gicheru**[§], **Linda Reiling**[†,‡], **Jack S. Richards**[†,‡], **Danny W. Wilson**[†], **Sash Lopaticki**[†], **Alan F. Cowman**[†], **Kevin Marsh**[§], and **James G. Beeson**[†,‡,¶]

[*]Karolinska Institutet, Microbiology, Tumor and Cell Biology, 17177 Stockholm, Sweden

[†]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia

[‡]The Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia

[§]Centre for Geographic Medicine Research, Coast, Kenya Medical Research Institute, Kilifi, Kenya

[¶]Department of Microbiology, Monash University, Victoria, Australia

Abstract

Antibodies that inhibit *Plasmodium falciparum* invasion of erythrocytes form an important component of human immunity against malaria, but key target antigens are largely unknown. Phenotypic variation by *P. falciparum* mediates the evasion of inhibitory antibodies, contributing to the capacity of *P. falciparum* to cause repeat and chronic infections. However, antigens involved in mediating immune evasion have not been defined and studies of the function of human antibodies are limited. In this study, we used novel approaches to determine the importance of *P. falciparum* erythrocyte-binding antigens (EBAs), which are important invasion ligands, as targets of human invasion inhibitory antibodies and define their role in contributing to immune evasion through variation in function. We evaluated the invasion-inhibitory activity of acquired antibodies from malaria-exposed children and adults from Kenya using *P. falciparum* with disruption of genes encoding EBA140, EBA175, and EBA181, either individually or combined as EBA140/EBA175 or EBA175/EBA181 double knock-outs. Our findings provide important new evidence that variation in the expression and function of the EBAs plays an important role in evasion of acquired antibodies and that a substantial amount of phenotypic diversity results from variation in expression of different EBAs that contributes to immune evasion by *P. falciparum*. All three EBAs were identified as important targets of naturally-acquired inhibitory antibodies demonstrated by differential inhibition of parental parasites greater than EBA knockout lines. This knowledge will help to advance malaria vaccine development and suggests that multiple invasion ligands need to be targeted to overcome the capacity of *P. falciparum* for immune evasion.

Keywords

EBA; immunity; malaria; PfRh; *Plasmodium falciparum*

Corresponding authors: Kristina E M Persson, Karolinska Institutet, Microbiology, Tumor and Cell Biology, 17177 Stockholm, Sweden, phone +46-852484509 kristina.persson@ki.se James G Beeson, Burnet Institute, Melbourne, Australia. Ph: +61-3-9282-2111, beeson@burnet.edu.au.

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Introduction

Malaria due to *Plasmodium falciparum* is a major cause of morbidity and mortality globally, with up to one million deaths each year (1). Malaria disease develops during the blood-stage of infection, when the merozoite form of the parasite invades erythrocytes and replicates inside them. After repeated exposure to *P. falciparum* infection, natural immunity is acquired that appears to prevent clinical symptoms by controlling blood-stage parasite replication (2, 3). This provides a strong rationale that the development of an effective malaria vaccine is achievable (4). Antibodies are an important component of acquired human immunity against malaria (5), and key targets of these antibodies include antigens expressed by merozoites (4). Antibodies that target merozoite antigens are believed to be important in mediating both acquired immunity and immunity generated by candidate blood-stage vaccines (6-9) and function, in part, by directly inhibiting invasion of erythrocytes (6, 7, 10, 11). However, there is a limited understanding of the targets of functionally important human antibodies and very few studies on these responses. *P. falciparum* can cause repeated and chronic infections due to its capacity for immune evasion, which has significant implications for vaccine development. However, the molecular basis for evasion of immune responses targeting merozoite antigens is unclear.

Merozoites can use different pathways, defined by receptor-ligand interactions, for invasion of erythrocytes and recent studies have suggested that this capacity for phenotypic variation contributes to immune evasion by *P. falciparum* (12). Using different parasite clones that varied only in their invasion phenotype, it was shown that changes in invasion pathways used by the merozoite influenced the susceptibility of *P. falciparum* to human invasion-inhibitory antibodies (12). The molecular basis for this immune evasion remains undefined, however, the use of alternate invasion pathways appears to primarily result from variation in the expression and/or use of members of two invasion ligand families, the erythrocyte binding antigens (EBAs) and *P. falciparum* reticulocyte-binding homologues (PfRh) (13-29). These protein families play essential roles in invasion, but the degree of functional redundancy among them means that not all ligands are required for invasion. Diversity in invasion phenotypes and variation in the expression and use of the EBA and PfRh proteins and has been demonstrated among clinical isolates in different populations (14, 22, 30-32) and using defined laboratory-adapted clones of *P. falciparum* (25, 28, 33, 34).

The EBAs are located in the micronemes and include EBA175, EBA140 (also known as BAEBL), EBA181 (also known as JESEBL), and EBL1 (35-37). The PfRh proteins are located in the rhoptries and include PfRh1, 2a, 2b, 4 and 5 (23, 25, 38-40). Additional members of these families, EBA165 and PfRh3, occur as pseudogenes (25, 41, 42). Invasion phenotypes can be broadly classified into two main pathways: i) sialic acid (SA)-dependent invasion, demonstrated by poor invasion of neuraminidase-treated erythrocytes (neuraminidase cleaves SA on the erythrocyte surface), and ii) SA-independent invasion, demonstrated by efficient invasion of neuraminidase-treated erythrocytes. SA-dependent (neuraminidase-sensitive) invasion involves the EBAs, and PfRh1 (15, 17-19, 23, 24, 28, 43, 44). EBA175 and EBA140 bind to the erythrocyte surface molecules glycophorin A (43-45) and C (19), respectively. EBA181 binds to SA on the erythrocyte surface and to band 4.1 protein (18, 46). EBL1 appears only to be expressed by some isolates, and can bind glycophorin B (37). PfRh1 binds SA residues on erythrocytes, but the specific receptor is unknown (23, 28). PfRh2 and PfRh4 are important in SA-independent invasion (17, 25, 33), but PfRh2 may also play a role in SA-dependent invasion (47-49). The two forms of PfRh2 are identical for approximately 80% of the N-terminal region (17). The receptor for PfRh2 is unknown but PfRh4 binds to complement receptor 1 on the surface of erythrocytes (34).

PfRh5 was recently shown to bind to the erythrocyte protein basigin (50) and is thought to be essential for invasion, but unlikely to play a role in phenotypic variation (51).

The EBAs and PfRh ligands are considered promising vaccine candidates due to their important functional roles; however, their importance as targets of acquired protective responses in humans has not been established, and the significance of the variation in EBA and PfRh expression for immune evasion is unknown. Antibodies are acquired to the EBAs and PfRh proteins through natural exposure (12, 52-55), and antibodies to EBA175, EBA140, EBA181, PfRh2, and PfRh4 (53-57) were found to be strongly associated with protective immunity in prospective longitudinal studies of children. Immunization of experimental animals with recombinant EBAs and PfRh proteins can generate antibodies that inhibit erythrocyte invasion *in vitro* (19, 58-64).

Dissecting the importance of specific antigens as targets of inhibitory antibodies in humans is challenging because antibodies to multiple antigens are co-acquired through natural exposure, making it difficult to attribute inhibitory activity to specific antigens. Additionally, comparing the inhibitory activity of human antibodies to isolates with different invasion phenotypes is complicated by other differences between isolates, such as polymorphisms in specific antigens that can influence the inhibitory activity of antibodies (65, 66). In this study, we used new approaches to understand the significance of the EBAs in immune evasion and as targets of immunity. To do this, we tested the ability of acquired antibodies from malaria-exposed African children and adults to inhibit invasion of *P. falciparum* isolates that had targeted disruption of EBA175, EBA140, or EBA181, or isolates with disruption of two EBAs in combination, compared to parental parasites. Changes in the susceptibility to human inhibitory antibodies resulting from disrupted EBA expression would point to the importance of phenotypic variation in EBA function in contributing to immune evasion and the importance of the EBAs as targets of inhibitory antibodies. Additionally, we evaluated the presence of antibodies specific to EBA and PfRh ligands in the study population. The findings from these studies are significant for advancing our understanding of immune evasion by *P. falciparum,* and have major implications for the malaria vaccine design and development.

Materials and Methods

Invasion inhibition assays

All sera were dialyzed to remove non-specific inhibitors, run in duplicates, 1/10 dilution in two separate assays with synchronized (sorbitol used every second day for 2 weeks before starting the assay) *P. falciparum* 3D7wt, 3D7ΔEBA175, 3D7ΔEBA140, 3D7ΔEBA181, 3D7ΔEBA175/181, 3D7ΔEBA140/175, W2mefwt, and W2mefΔEBA140 parasites (67). For a subset of samples, the inhibitory effect of treated samples was confirmed by immunoglobulin purified from the same samples (67). Rabbit antibodies to $MSP1_{19}$ were kindly provided by Brendan Crabb (68). Samples from non-exposed donors were used as negative and polyclonal anti-AMA1 antibodies as positive controls in all assays. Samples were tested with all the different lines in parallel in the same experiments. A difference in invasion between the lines of >25% in invasion was designated as the cut-off for differential inhibition on the basis that this was greater than the variance seen in the assays, and that a difference of >25% was likely to be biologically significant; in repeated testing of non-immune control samples in our assays, two standard deviation from the mean was 10-15%. We have previously demonstrated that these assays have a high level of reproducibility (11, 67, 69). Preadsorption of treated sera against erythrocytes did not alter their inhibitory activity. A selection of sera was also tested for antibodies to the surface of uninfected erythrocytes maintained in culture (70); there was very little reactivity against normal

erythrocytes and there was no relationship between antibody binding to erythrocytes and invasion inhibitory activity.

Antibodies to recombinant proteins by ELISA

Standard ELISAs were performed (12). For each serum, the absorbance from wells containing GST only was deducted. Recombinant proteins used were EBA140 RIII-V 3D7 allele (aa746-1045) (19), EBA175 RIII-V W2mef and 3D7 alleles (aa 761-1271) (71), EBA181 RIII-V 3D7 allele (aa755-1339) (18), PfRh4 3D7 allele (aa1160-1370) (25) and PfRh2 3D7 allele (aa2027-2533) (17), as well as schizont extract (3D7 allele) (12).

Study population and sera samples

Sera samples from 18 adults (47 (18-81) years, 22.2% male, 16.7% *P. falciparum* positive) and 53 children (median age (range) 8 (2-12) years, 60.4% male, 49.1% *P. falciparum* positive) were randomly selected from a community-based cross-sectional survey of residents in Ngerenya in the Kilifi District, Kenya, in September 1998, which immediately preceded a period of increased malaria transmission (71). We also obtained 31 samples from children in Ngerenya (median age 5 (0-8) years, 41.9% male, 12.9% *P. falciparum* positive) collected in May 2003, at the start of the rainy season associated with higher malaria transmission. These samples were selected from a larger cohort based on their inhibitory activity against 3D7wt. Ngerenya is an area with transmission of malaria mainly during two annual rainy seasons (May and October/November). We also used randomly selected sera from 28 adult, anonymous blood donors collected in May 2004 in Kilifi, and as controls non-exposed adult residents in Australia/UK (n=40). Ethical approval was obtained from the Ethics Committee of the Kenya Medical Research Institute, Nairobi, Kenya and from the Walter and Eliza Hall Institute Ethics Committee, Melbourne, Australia, and Alfred Hospital Human Research and Ethics Committee (for the Burnet Institute). All samples were obtained after written informed consent.

Statistical analysis

Differences in invasion between knock-out and 3D7wt were assessed using paired t-tests separately for each population sample set. The association of invasion with age and parasitaemic status was assessed using t-tests or Mann-Whitney *U* test where appropriate. Differences in the frequency of high and low invasion inhibitors with the sample set was assessed using chi-squared or Fisher's exact tests where appropriate. The correlation between IgG responses to EBAs/PfRhs and invasion inhibition results was assessed by Spearman's rank correlation using all ELISA values. SPSS (for Windows Rel 16.0. 2007. Chicago:SPSS Inc) was used for statistical analysis.

Results

Phenotypic variation and evasion of inhibitory antibodies

To study the function of the EBA proteins in phenotypic variation for immune escape and as targets of human inhibitory antibodies, we used 3D7 parasite lines that had targeted disruption of single EBA genes (EBA140, EBA175, or EBA181) and 3D7 parasites with disruption of two EBA genes: EBA140/EBA175, or EBA175/EBA181 (61). Previously, transcriptional analysis of these lines revealed significant up-regulation in PfRh4 gene expression in 3D7ΔEBA175, 3D7ΔEBA140/175 and 3D7ΔEBA175/181 compared to the 3D7 parental line (61). Otherwise, there were no significant changes in merozoite gene expression. In 3D7ΔEBA140, there were no changes in transcriptional levels of these genes compared to 3D7 parental parasites.

Human serum antibodies were tested for their ability to inhibit invasion of parasites with targeted disruption of EBA protein expression versus parental parasites. Differential inhibition by acquired antibodies of parasites that differ only in the presence or absence of a specific EBA protein would suggest it plays a role in immune evasion and in defining the inhibitory or antigenic phenotype of parasites. Serum antibodies were tested from individuals residing in a malaria-endemic region of Kenya for inhibitory activity of merozoite invasion. Four sets of samples were used to represent different ages and levels of exposure to malaria. Samples from children and adults were collected at the same time in the Ngerenya community in 1998. Samples from Ngerenya children were also collected at a later time when malaria transmission was reduced (2003), and also from adults around the Kilifi township in 2004. For these studies, differential inhibition was regarded as significant if there was a difference of at least 25% in the extent of inhibition between the comparison lines (12).

Testing human antibodies in invasion inhibition assays, we found that the disruption of EBAs had a major effect on the susceptibility of parasites to inhibitory antibodies when comparing inhibition of the EBA knock-out lines versus the parental parasites. This was seen among all samples sets examined (Table I; Fig. 1 and 2). The effect was greatest with 3D7ΔEBA140 and 3D7ΔEBA175 for which 84% of samples (for both lines) gave differential inhibition of knock-out versus parental parasites. Interestingly, for 3D7ΔEBA140 most of the samples with differential inhibition showed greater inhibition of parental parasites compared to the knock-out parasites in all sample sets, suggesting that antibodies were directed against EBA140, and that absence of EBA140 function led to escape from inhibitory antibodies for most samples. Reflecting this, the mean inhibition of 3D7ΔEBA140 by all samples was significantly lower ($p<0.05$) than 3D7 (Fig. 2). In contrast, for 3D7ΔEBA175 there was a very different phenotype with significantly greater ($p<0.05$) mean inhibition of the knock-out compared to parental parasites by all study samples, and a substantial proportion of samples inhibited 3D7ΔEBA175 greater than 3D7 parental parasites. This suggests that the phenotypic change induced by loss of EBA175 function led to greater antibody inhibitory activity against the knock-out parasite, possibly due to antibodies targeting ligands that were replacing the function of EBA175, such as PfRh proteins. Lack of EBA181 function also had a significant effect on susceptibility to inhibitory antibodies, with 40% of samples showing differential inhibition of 3D7ΔEBA181 compared to 3D7wt. Most samples showed greater inhibition of the knock-out compared to the parental parasites, as was seen with 3D7ΔEBA175, but this effect was not as great as that seen with 3D7ΔEBA175; mean inhibition of 3D7ΔEBA181 by all samples was significantly greater ($p<0.05$) than 3D7wt. Comparing the pattern of inhibitory activity against the different lines by individual samples suggests there is also substantial diversity in the repertoire of responses seen among individuals. Individual samples varied in the extent and direction of inhibition of mutant versus parental parasite lines (Fig. 3; Supplementary Material, Fig. S1).

Prior studies have shown that the function and binding affinity of EBA140 can vary substantially between different isolates, which is associated with polymorphisms in EBA140 (19). Therefore, we investigated whether the genetic background of the parasite influenced the activity of human inhibitory antibodies to EBA140 by testing human antibodies for differential inhibition of the W2mef parasite with disruption of EBA140. In the W2mef isolate, EBA140 displays lower erythrocyte-binding activity and appears less important in invasion compared to its role in the 3D7 isolate. Reflective of this functional difference, deletion of EBA140 in the W2mef isolate had a much less marked effect on antibody activity than seen in 3D7. Only 1.5% of the samples we tested differentially inhibited W2mefΔEBA140 versus W2mef parental parasites, and the mean±SD level of invasion in the presence of serum antibodies was very similar for the two isolates (77%±18% in

W2mefΔEBA140 and 73±16% for W2mefwt). This is consistent with EBA140 being less important in invasion in W2mef versus 3D7 isolates, and indicates that the genetic background of the parasite line influences the importance of EBA140 as an antibody target and for phenotypic variation to mediate immune evasion.

These findings demonstrate that changes in the expression of EBA175, EBA140, or EBA181 substantially impact on the antigenic properties of merozoite invasion and the susceptibility to acquired inhibitory antibodies, and that there are differences in the antigenic properties of the different EBA knockout parasites. As controls in these studies, we tested the mutant and parental parasite lines for inhibition by antibodies raised against MSP1-19, the expression of which does not vary between isolates, or by heparin (1 mg/ml), which specifically inhibits erythrocyte invasion (72). The level of inhibition was very similar between the parental and different knock-out lines (supplementary material, Fig. S2). Furthermore, we included serum antibodies from malaria-naïve donors and they gave no differential inhibition of the different parasite lines, in contrast to the findings using samples from Kenyan donors. There were no consistent associations between active parasitemia in donors and the level of invasion inhibition by samples. However, few individuals were parasitemic at the time of sample collection and the study was not designed to detect this association.

Inhibition of parasites with deletion of two EBAs

We next tested the impact of disruption of two different EBAs (double knock-out parasites) on inhibitory antibodies compared to 3D7 parental parasites (parasite lines 3D7ΔEBA140/175 and 3D7ΔEBA175/181; Fig. 1-3). Differential inhibition of the two double knock-out isolates compared to parental parasites was seen in a substantial proportion of samples, and in all sample sets; overall 38% and 32% of samples showed differential inhibition of 3D7ΔEBA140/175 and 3D7ΔEBA175/181, respectively (Fig. 1; Table I). For the double knock-out parasite lines, there was little overall difference in the median inhibition of the parental versus knock-out parasites by all samples (Fig. 2) because there were similar numbers of samples that gave greater or lower inhibition of the knock-out parasites compared to parental. These results suggest that disruption of two EBA genes led to substantial changes in the susceptibility to inhibitory antibodies; however, it did not result in a more pronounced phenotypic change compared to single EBA knock-outs.

To determine whether the antigenic phenotypes of the single and double KO lines were different, we tested samples for differential inhibition of the various mutant parasite lines (Fig. 4). A large proportion of samples (65%) gave differential inhibition of 3D7ΔEBA140 compared to 3D7ΔEBA140/175 double knockout, clearly indicating that the antigenic properties of these two lines and the pattern of susceptibility to inhibitory antibodies were very different. There was also significant differential inhibition of 3D7ΔEBA181 versus 3D7ΔEBA175/181 (21% of samples), suggesting the phenotypes of these isolates differ. In contrast, there was little differential inhibition of 3D7ΔEBA175 versus either of the double knockout lines, suggesting that the antigenic properties and phenotypes of these lines are similar. Overall, the 3D7ΔEBA175/181 and 3D7ΔEBA175/140 double knockouts appear to be more similar to the EBA175 single knockout in their antigenic properties, whereas the phenotypes of the 3D7ΔEBA140 and 3D7ΔEBA181 appear to be distinct from the other knock-out lines and from parental parasites.

Relatedness between antibody responses and antigenic differences between parasites

To better understand the acquisition of immunity and antigenic differences between the different EBA-knockout parasite lines, we examined the relationship between inhibitory antibodies to the different isolates. We first examined whether samples that showed less

inhibition of one EBA knock-out line also showed less inhibition of other EBA knock-out parasites, compared to parental parasites (Table II). These analyses suggested that inhibitory antibody responses to different isolates were not strongly related. For example, of the many samples that showed less inhibition of 3D7ΔEBA140 compared to 3D7wt parasites, only a small proportion showed less inhibition of 3D7ΔEBA175 (16.2% of all samples), 3D7ΔEBA181 (3.1%), 3D7ΔEBA140/175 (15.4%) or 3D7ΔEBA175/181 (19.2%) compared to parental parasites. We next determined whether samples that showed greater inhibition of one EBA knock-out line also gave greater inhibition of other EBA knock-out parasites, compared to the parental line (Table II). Again, these inhibitory antibody responses were not highly related between isolates. For example, of the many samples that inhibited 3D7ΔEBA175 more than 3D7wt parasites, only a small proportion showed greater inhibition of 3D7ΔEBA181 (19.1% of all samples), 3D7ΔEBA140/175 (11.8%), or 3D7ΔEBA175/181 (4.4%), compared to parental parasites; no samples showed this pattern of inhibition for both 3D7ΔEBA175 and 3D7ΔEBA140 parasites.

Overall, these analyses suggest that inhibitory antibody responses were not highly related to each other in the study samples and that each of the EBA single knock-outs led to distinct antigenic or inhibitory phenotypes, as defined by the susceptibility to inhibitory antibodies. However, as noted earlier, the inhibitory phenotypes and antigenic properties of the 3D7ΔEBA175/181 and 3D7ΔEBA175/140 double knockouts appeared similar to 3D7ΔEBA175. Overall, these analyses further support the concept that variation in the use or expression of the EBA proteins, which is observed among clinical isolates, creates a substantial amount of phenotypic diversity that alters susceptibility to human antibodies and contributes to immune escape.

Inhibitory antibodies target SA-dependent and SA-independent invasion pathways

We next aimed to understand the extent to which invasion-inhibitory antibodies target ligands of SA-dependent and SA-independent invasion pathways, and the EBAs more specifically. The parasite line 3D7 invades erythrocytes using ligands of both the SA-dependent (including EBA175, EBA140, and EBA181) and SA-independent (such as PfRh2 and PfRh4) pathways.

Reduced invasion inhibition by serum samples of the EBA knock-out lines compared to parental parasites suggests the presence of antibodies to the EBA proteins (and possibly other ligands of SA-dependent invasion). A large proportion of samples (82%) showed greater inhibition of the parental parasites compared to the 3D7ΔEBA140 parasites, suggesting that inhibitory antibodies target EBA140. The 3D7ΔEBA140 showed significantly less inhibition compared to 3D7wt in all four sample sets: Kilifi adults, Ngerenya 1998 adults, Ngerenya 1998 children, and Ngerenya 2003 children (Fig. 1-2, $p \leq 0.001$ for all differences). With other EBA knock-out parasite lines, there were fewer samples with this effect; 13% inhibited 3D7wt more than 3D7ΔEBA175 parasites, and only 5% inhibited 3D7wt parasites more than the 3D7ΔEBA181. This might suggest that EBA175 and EBA181 are less important targets of inhibitory antibodies in 3D7, or that a change in invasion phenotype may occur with the loss of EBA175 or EBA181 that leads to a greater susceptibility to other inhibitory antibodies that target ligands replacing the function of EBA175 or EBA181. For the double knock-outs, 16% and 21% inhibited the parental more than 3D7ΔEBA140/175 and 3D7ΔEBA175/181, respectively, further supporting the conclusion that some individuals have inhibitory antibodies to the EBAs, and possibly other ligands of SA-dependent invasion.

Inhibition of the EBA knock-out lines more than the parental parasites suggest that inhibitory antibodies may also target ligands of SA-independent invasion, such as PfRh2 and PfRh4, particularly when comparing the double knock-outs to parental parasites. This

pattern of greater inhibition of knockout versus parental parasites was seen for 71% and 35% of samples for 3D7ΔEBA175 and 3D7ΔEBA181, respectively, but was uncommon for 3D7ΔEBA140 (1.5% of samples). Greater inhibition of 3D7ΔEBA140/175 and 3D7ΔEBA175/181 compared to parental parasites was seen among 11% and 22% of samples (Table I). Disruption of EBA genes, particularly EBA175, leads to a greater reliance on SA-independent ligands for efficient invasion of erythrocytes (25, 59). Therefore, greater inhibition of knockout lines compared to parental parasites suggest inhibitory antibodies target these ligands, and previous studies have shown that affinity-purified human antibodies to PfRh4 can inhibit SA-independent invasion (54); however, further studies are needed to understand the importance of ligands of SA-independent invasion as targets of human inhibitory antibodies.

Presence of antibodies to EBA and PfRh invasion ligands

Differential inhibition by human antibodies of EBA knock-out parasites compared to parental parasites suggests that the EBA and PfRh ligands are important targets of antibodies. To assess this, we measured IgG responses to recombinant EBA and PfRh antigens by ELISA in the different sample sets (Fig. 5). Results confirm that antibodies to EBA175, EBA140, and EBA181 are common in the study population although antibodies to EBA181 were lower in the Ngerenya 2003 children. Antibodies to PfRh2 and PfRh4, as representative examples of SA-independent invasion ligands, were also common, although the prevalence of antibodies to PfRh4 was more variable across the samples sets than PfRh2. In general, adults showed higher levels of antibodies compared to children's samples. There was little or no reactivity of sera from malaria-naïve individuals. Antibody levels to different EBA and PfRH proteins were also significantly positively correlated with each other, suggesting the co-acquisition of antibodies to multiple invasion ligands following *P. falciparum* exposure. Significant correlations were seen between antibodies to different EBAs (range of r_s values 0.63 to 0.78; $p<0.01$ for all), PfRh2 and PfRh4 ($r_s=0.58$; $p<0.01$), and between EBAs and PfRh proteins (range of r_s values 0.47 to 0.73; $p<0.01$ for all).

We also examined the relationship between antibodies to EBA and PfRh ligands and differential invasion inhibition of parasites. Considering the correlation between antibodies to different invasion ligands by ELISA, the ability to dissect the relationship between any antigen-specific response by ELISA and specific invasion inhibitory activity was limited. Greater inhibition of 3D7 parental parasites than 3D7ΔEBA140 suggested the presence of inhibitory antibodies targeting EBA140. Consistent with this, there was a significant correlation, of moderate strength, between antibodies to EBA140 by ELISA and the extent of differential inhibition of 3D7 parental parasites compared to 3D7ΔEBA140 ($r_s=0.27$; $p<0.05$). There was a similar finding for EBA181, with antibodies to EBA181 by ELISA being significantly correlated with the extent of differential inhibition of 3D7 parental parasites compared to 3D7ΔEBA181 ($r_s=0.32$; $p<0.05$). This suggests that antibodies measured by ELISA to these recombinant proteins are broadly reflective of functional inhibitory activity, but are not strongly predictive of ligand-specific inhibitory activity of serum antibodies. Few samples showed differential inhibition of 3D7 parental parasites greater than 3D7ΔEBA175 (Figure 1); to the contrary, most samples gave greater inhibition of 3D7ΔEBA175 compared to 3D7 parental, suggesting the presence of inhibitory antibodies targeting ligands of SA-independent invasion, which includes PfRh2 and PfRh4, because disruption of EBA175 leads to a greater reliance on SA-independent ligands for invasion (25, 59). Consistent with this, antibodies to PfRh2 were significantly correlated with greater inhibition of 3D7ΔEBA175 parasites compared to 3D7 parental parasites ($r_s=0.42$; $p<0.01$), especially in the Ngerenya 2003 children's samples ($r_s=0.53$, $p<0.01$). This was consistent with data showing that disruption of EBA175 leads to a greater reliance on ligands of SA-independent invasion, such as PfRh2 (17, 24, 25, 73). There was also a correlation between

antibodies to PfRh2 and inhibition of the double knockout parasites 3D7ΔEBA140/175 (r=0.47, p<0.01 in Ngerenya children 2003; r=0.29, p<0.05 in Ngerenya children 1998, r=0.76 p<0.001 in Ngerenya 1998 adults) and 3D7ΔEBA175/181 (r=0.45, p<0.001 in Ngerenya children 1998, r=0.50 p<0.1 in Ngerenya 1998 adults) compared to 3D7 parental, consistent with these parasites also being more reliant on ligands of SA-independent invasion.

Discussion

The identification of major targets of protective immune responses and understanding mechanisms of immune evasion that enable chronic and repeated infections over time are key issues in malaria immunity and vaccine development. In this study we aimed to determine whether all of the EBA ligands are important targets of invasion inhibitory antibodies, and whether variation in the expression/use of EBAs contributes to immune evasion. To achieve this we used genetically modified parasites with targeted disruption of EBA protein expression, individually or in combination, in assays that measure human invasion inhibitory antibodies. The advantage of assays employing live parasites is that the antigens are correctly folded and presented in their native context, and antibodies are measured to all epitopes presented on the protein rather than assessing antibodies only to specific domains or regions as is regularly done in ELISA assays.

Our striking findings that disrupting the function of individual EBAs dramatically alters the susceptibility to human inhibitory antibodies provide important new insights into the molecular basis of evasion of human immune responses by *P. falciparum*. A substantial proportion of serum samples showed differential inhibition of parasites with disruption of EBA expression compared to parental parasites, indicating that changes in antigenic properties occur with changes in EBA function. Disruption of each of EBA140, EBA175, and EBA181 led to major changes in susceptibility to inhibitory antibodies indicating that all three EBAs can contribute to immune evasion through variation in their function. Furthermore, our findings strongly suggest that each of the three knock-out lines and the parental parasites are antigenically-distinct from each other, as defined by the inhibitory profile against our panel of human antibodies. This indicates that there is a significant level of antigenic diversity that could contribute to immune evasion. Differences in the inhibitory activity of antibodies against genetically different isolates have been long known and believed to be important in immune evasion (11, 70, 71, 74) but the underlying molecular mechanisms have remained unclear, in part due to a lack of tools to dissect specific responses. Our findings illustrate the value of translating new molecular approaches to studies of clinical immunology.

Substantial polymorphism is present in the erythrocyte binding region of EBA175, but is less marked in EBA140 or EBA181. The significance of these polymorphisms in EBA175 for evasion of inhibitory antibodies has not been clearly established. Studies using antibodies raised in rabbits suggest that polymorphisms in the binding region of EBA175 do not have a major impact on the invasion inhibitory activity of antibodies (76, 77). However, we did demonstrate here that the genetic background of the parasite influences the importance of EBA140 as an antibody target and its role in phenotypic variation to mediate immune evasion. While EBA140 appeared to be an important target of inhibitory antibodies with the 3D7 isolate, it appeared much less important in the W2mef isolate. This is consistent with previous studies suggesting that EBA140 is less important in invasion with the W2mef isolate. The W2mef EBA140 polymorphic variant has a reduced erythrocyte binding activity compared to 3D7 EBA140 even though the expression of protein appears similar between isolates (75). The EBA140 sequence differs in three amino acids between 3D7 and W2mef, (in the F1 domain). Furthermore, antibodies to EBA140 generated in

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rabbits gave much greater inhibition of invasion with 3D7 parasites than W2mef parasites (19, 75), consistent with our findings here using human antibodies, and indicating that EBA140 has a less prominent role in the invasion of the W2mef isolate.

The differential inhibition of parental parasites more than knockout lines that we observed indicates that all three EBAs appear to be targets of human inhibitory antibodies. A majority of samples showed reduced inhibition of 3D7ΔEBA140 compared to 3D7wt, suggesting inhibitory antibodies to EBA140 are prominent in this population. It has been shown previously that deletion of the EBA140 gene does not lead to any significant changes in levels of mRNA for EBA175, EBA181, PfRh2, PfRh4 (61), indicating that the differential inhibition seen is not due to that action of antibodies against other EBAs or PfRhs.

Fewer samples demonstrated reduced inhibition of 3D7ΔEBA175 compared to 3D7wt, reflecting the presence of inhibitory antibodies to EBA175 in the population. It is possible that EBA175 is a less important target of inhibitory antibodies with the 3D7 parasite line we used, compared to EBA140. Alternatively, the loss of EBA175 may lead to a wider change in the invasion phenotype and lead to a greater reliance on other invasion ligands, such as EBA140 or PfRh ligands that are prominent targets of inhibitory antibodies. The importance of the EBAs as targets of inhibitory antibodies was supported by results showing that antibodies to recombinant EBAs by ELISA were highly prevalent in the population, and there were some significant correlations between antibodies to recombinant EBA140 and EBA181 proteins and differential inhibition of parental versus knockout parasite lines. However, our results suggest that measuring antibodies to recombinant proteins by ELISA cannot be relied upon as a proxy for functional antibody activity. Future studies with full-length or native proteins may help understand the relationship between antibody levels versus function; however, standard immunoassays cannot account for antibody affinity and epitope specificity that are likely to be important for functional activity. This highlights the value of using functional assays to complement data obtained from standard immunoassays.

Our findings also suggest that ligands of SA-independent invasion, such as PfRh2 and PfRh4, are important targets of inhibitory antibodies. This was demonstrated by greater inhibition by human antibodies of 3D7ΔEBA175 (or EBA175/140 and EBA175/181 double knockouts) versus 3D7wt parasites. Compared to 3D7wt, 3D7ΔEBA175 has increased expression of PfRh4 (61) and has a neuramindase-resistant invasion phenotype (59), suggesting a greater reliance on ligands of SA-independent invasion compared to parental parasites. In contrast, PfRh4 expression was unchanged in 3D7ΔEBA140, which had less inhibition of by human antibodies compared to parental parasites. Consistent with this conclusion, acquired antibodies were reactive to recombinant PfRh2 and PfRh4 in the population, as measured by ELISA, and the level of antibodies to PfRh2 were significantly correlated with differential inhibition of 3D7ΔEBA140/175 or 3D7ΔEBA175 compared to 3D7wt parasites. Recent studies have reported that antibodies to PfRh2 and PfRh4 are associated with protective immunity, and that affinity purified antibodies to PfRh4 from malaria-exposed donors can inhibit invasion at physiologically relevant concentrations (53, 54). Many individuals may have functional antibodies to both EBAs and PfRh ligands, which may explain why we did not observe significant differential inhibition of 3D7ΔEBA175 or 3D7ΔEBA140/175 compared to 3D7wt among some samples.

The EBAs are considered attractive vaccine candidates because of their important biological role in invasion, and their relatively limited level of polymorphism (4). Our results suggest that the amount of antigenic diversity at the level of erythrocyte invasion, mediated by variation in the use/expression of the EBAs and PfRhs, is substantial, and this strongly suggests that a vaccine based on any of the EBAs would require the inclusion of multiple antigens to overcome the parasite's capacity for immune evasion, such as a combination of

EBA and PfRh ligands (12). While most research on the EBAs has focussed on EBA175 as a potential subunit vaccine candidate, our results highlight EBA140 as an important target of inhibitory antibodies and this antigen needs to be further studied as a potential vaccine candidate. Recent reports suggest that vaccine-induced antibodies raised in rabbits against PfRh5, or its binding partner, can inhibit multiple parasite lines, and may represent attractive vaccine candidates (51, 78). However, the significance of these antigens as targets of acquired human immunity is not known.

In conclusion, we have provided new insights into the molecular basis for evasion of human immune responses by *P. falciparum,* providing evidence that variation in the function/expression of the EBAs contributes to immune evasion and that the EBAs are important targets of human invasion-inhibitory antibodies. The importance of the EBAs supports their development as promising vaccine candidates; however, the demonstration here of their role in immune evasion through phenotypic variation suggests that vaccines would need to target multiple invasion ligands in order to counter parasite immune evasion and be highly effective. Therefore, our results have important implications for malaria vaccine development and understanding human immunity against malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Pf	Plasmodium falciparum
EBA	erythrocyte-binding antigen
PfRh	*Plasmodium falciparum* reticulocyte-binding homologue
wt	wild-type
SA	sialic acid

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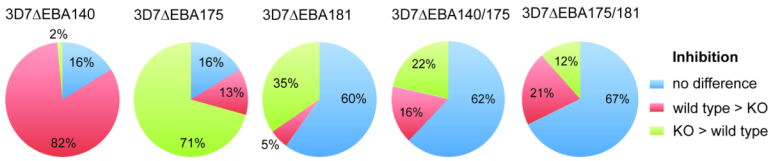

FIGURE 1. Differential inhibition of *P. falciparum* lines by serum antibodies from malaria-exposed Kenyan individuals

Results show the proportion of samples (n=130) that differentially inhibited the invasion of 3D7wt parasites compared to 3D7 lines with disruption of specific EBA genes. Differential inhibition was considered significant if there was >25% difference in the level of inhibition between two parasite lines; 'no difference' was regarded as <25% difference in the level of inhibition (indicated in blue). The proportion of samples that inhibited the knock-out line more than the 3D7wt line is shown in green; the proportion of samples that inhibited 3D7wt more than the knock-out line is shown in red.

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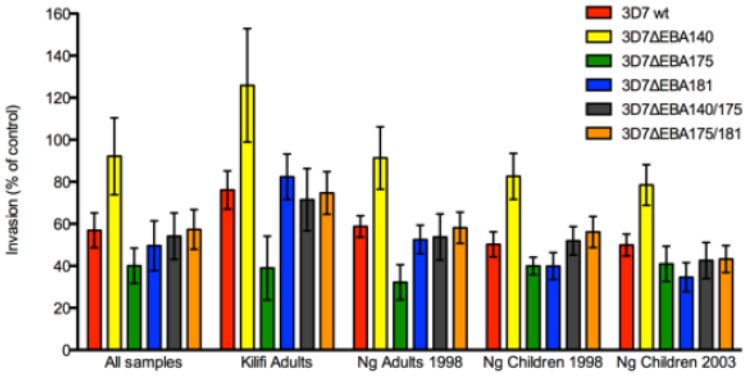

FIGURE 2. Mean of invasion inhibition by serum antibodies from Kenyan donors

Serum samples were tested against *P. falciparum* 3D7wt or 3D7 lines with disruption of EBA175, EBA140, or EBA181, both EBA140 and EBA175, or both EBA175 and EBA181. Results are shown for all samples tested, or grouped for each of the different sample sets used. Values are expressed relative to control samples from non-exposed donors. Values represent means and error bars show one standard deviation. Differences in mean invasion of the EBA knockout lines was significantly different ($p<0.05$) compared to 3D7wt parasites for all lines and samples subsets, except for 3D7ΔEBA175/140 with analysis of all samples, and Ngerenya adults and children 1998; 3D7ΔEBA181 for analysis of Ngerenya adults and children 1998; and 3D7ΔEBA140/175 for analysis of Ngereyan adults 1998.

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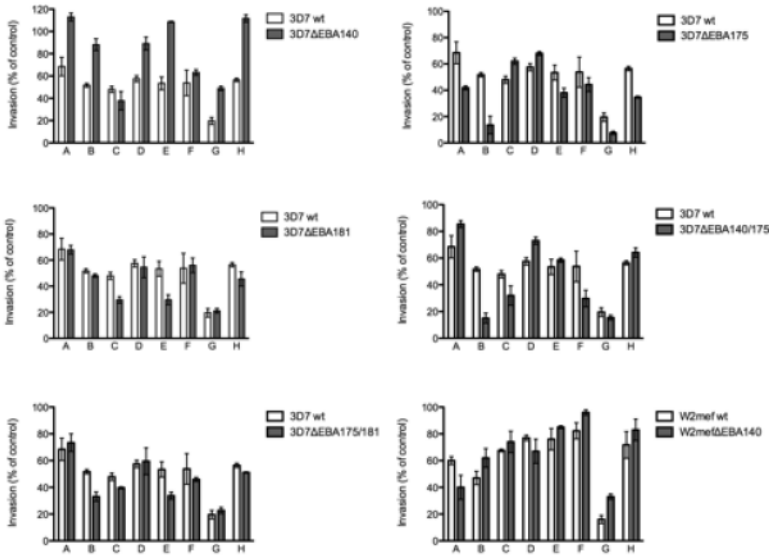

FIGURE 3. Inhibition of different *P. falciparum* lines by serum antibodies from malaria-exposed Kenyan individuals

Results show the effect of serum antibodies (from individuals A-H) on the invasion of the parental parasite line versus the knock-out line. Samples shown were selected to demonstrate representative examples of the inhibitory activities observed in the study. Values are expressed as percentage of invasion relative to non-exposed donors. All samples were tested in duplicate in two separate assays and values represent mean ± range.

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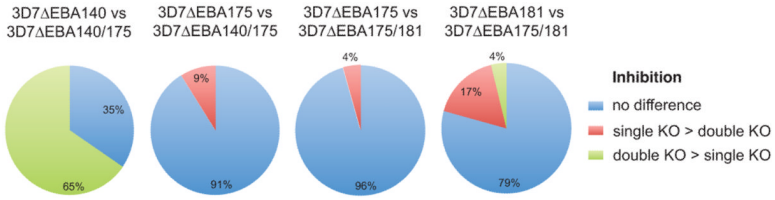

FIGURE 4. Differential invasion inhibition by human antibodies of *P. falciparum* lines with single versus double gene knock-outs

Results show the proportion of serum antibody samples (n=130) that differentially inhibited the invasion of 3D7 parasites with a single EBA knockout compared to 3D7 lines with disruption of two different EBAs (double knockouts). In red is the proportion of samples that inhibited the *in vitro* invasion of the 3D7 single knockout parasite line greater than the 3D7 double knock-out line, and in green is the proportion of samples that inhibited the invasion of 3D7 double knock-out parasite line more than the 3D7 single knock-out line. 'No difference' means <25% difference in inhibition (indicated in blue).

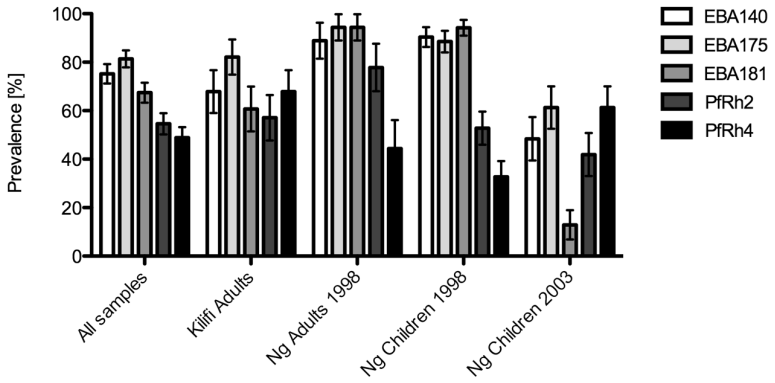

FIGURE. 5. Antibodies against recombinant EBA and PfRh proteins measured by ELISA in Kenyan individuals

Results show the prevalence of antibodies to recombinant EBA and PfRh proteins measured by ELISA among the different sample sets included in the study. Error bars represent standard error of a proportion. Antibody reactivity among malaria-naïve donors was negligible.

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TABLE I

Proportion of individuals with differential invasion inhibition of 3D7wt and 3D7 knock-out lines.

	ΔEBA140	ΔEBA175	ΔEBA181	ΔEBA140/175	ΔEBA175/181
Inhibition of 3D7wt>3D7-KO line[1]					
All Samples (n=130)	107 (82.3)	13 (13.1) *	7 (5.4)	21 (16.2)	27 (20.8)
Kilifi Adults 2006 (n=28)	23 (82.1)	N/A *	4 (14.3)	5 (17.9)	3 (10.7)
Ngerenya Adults 1998 (n=18)	13 (72.2)	3 (16.7) *	0 (0)	3 (16.7)	2 (11.1)
Ngerenya Children 1998 (n=53)	45 (84.9)	9 (17.0) *	2 (3.8)	11 (20.8)	21 (39.6)
Ngerenya Children 2003 (n=31)	26 (83.9)	1 (3.2)	1 (3.2)	2 (6.5)	1 (3.2)
Inhibition of 3D7-KO line > 3D7wt[2]					
All Samples (n=130)	2 (1.5)	7 (70.7) *	45 (34.6)	28 (21.5)	15 (11.5)
Kilifi Adults 2006 (n=28)	2 (7.1)	N/A *	0 (0)	6 (21.4)	3 (10.7)
Ngerenya Adults 1998 (n=18)	0 (0)	7 (43.8) *	4 (22.2)	7 (38.9)	2 (11.1)
Ngerenya Children 1998 (n=53)	0 (0)	31 (59.6) *	23 (43.4)	6 (11.3)	4 (7.5)
Ngerenya Children 2003 (n=31)	0 (0)	13 (41.9)	18 (58.1)	9 (29)	6 (19.4)

Values show the number of samples (n=130) with differential inhibition and the total number of samples tested (proportion %).

1) Results show the proportion of samples that inhibited the *in vitro* invasion of 3D7wt greater than the corresponding 3D7 knock-out line.

2) Results show the proportion of samples that inhibited the in vitro invasion of the 3D7 knock-out line greater than 3D7wt.

* sample numbers were n=99 (all samples), N/A samples not available (Kilifi Adults 2006), n=16 (Ng Adults 1998), n=52 (Ng Children 1998) due to limited sample availability.

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TABLE II

Relatedness between inhibitory antibodies: proportion of individuals showing differential invasion inhibition of two knock-out lines compared to 3D7wt.

	ΔEBA140	ΔEBA175	ΔEBA181	ΔEBA140/175
	n (%)	n (%)	n (%)	n (%)
Inhibition of 3D7wt>3D7-KOline[1]				
ΔEBA175	11 (16.2)			
ΔEBA181	4 (3.1)	1 (1.5)		
ΔEBA140/175	20 (15.4)	2 (2.9)	4 (3.1)	
ΔEBA175/181	25 (19.2)	4 (5.9)	3 (2.3)	10 (7.7)
Inhibition of 3D7-KOline>3D7wt[2]				
ΔEBA175	0 (0)			
ΔEBA181	0 (0)	13 (19.1)		
ΔEBA140/175	2 (1.5)	8 (11.8)	11 (8.5)	
ΔEBA175/181	0 (0)	3 (4.4)	5 (3.8)	9 (6.9)

Values represent number of samples and percent of total samples in brackets (percentages calculated with sample numbers of n=130 with the exception of 3D7ΔEBA175 where percentages represent n=68 (Ngerenya 1998 samples only)).

1) Samples that showed inhibition of the *in vitro* invasion of 3D7wt that was greater than the corresponding 3D7 knock-out line, for two different knock-out line comparisons.

2) Samples that showed inhibition of the *in vitro* invasion of a 3D7 knock-out line that was greater than 3D7wt, for two different knockout line comparisons.