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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 Lopatički,† Alan F. Cowman,‡ Kevin Marsh,§ and James G. Beeson†‡#

Abs that inhibit Plasmodium falciparum invasion of erythrocytes form an important component of human immunity against malaria, but key target Ags are largely unknown. Phenotypic variation by P. falciparum mediates the evasion of inhibitory Abs, contributing to the capacity of P. falciparum to cause repeat and chronic infections. However, Abs involved in mediating immune evasion have not been defined, and studies of the function of human Abs are limited. In this study, we used novel approaches to determine the importance of P. falciparum erythrocyte-binding Ags (EBAs), which are important invasion ligands, as targets of human invasion-inhibitory Abs and define their role in contributing to immune evasion through variation in function. We evaluated the invasion-inhibitory activity of acquired Abs 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 knockouts. Our findings provide important new evidence that variation in the expression and function of the EBAs plays an important role in evasion of acquired Abs 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 Abs 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. The Journal of Immunology, 2013, 191: 000–000.

Malaria due to Plasmodium falciparum is a major cause of morbidity and mortality globally, with ≤ 1 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 invasion, natural immunity is acquired and appears to prevent clinical symptoms by controlling blood-stage parasite replication (2, 3). This immunity provides a strong rationale for the idea that development of an effective malaria vaccine is achievable (4). Abs are an important component of acquired human immunity against malaria (5), and key targets of these Abs include Ags expressed by merozoites (4). Abs that target merozoite Ags 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, understanding of the targets of functionally important human Abs is limited, and very few studies on these responses are available. P. falciparum can cause repeated and chronic infections owing to its capacity for immune evasion, which has significant implications for vaccine development. However, the molecular basis for evasion of immune responses targeting merozoite Ags 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, a study showed that changes in invasion pathways used by the merozoite influenced the susceptibility of P. falciparum to human invasion inhibitory Abs (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 Ags (EBAs) and P. falciparum reticulocyte-binding homologs (PIRhs) (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 have been demonstrated among clinical isolates in different populations (14, 22, 30–32) as well as in 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: 1) sialic acid (SA)–dependent invasion, demonstrated by poor invasion of neuraminidase-treated erythrocytes (neuraminidase cleaves SA on the erythrocyte surface), and 2) 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 glycophrin 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 to be expressed only by some isolates and can bind glycophrin 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 ~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 owing 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. Abs to the EBAs and PfRh proteins are acquired through natural exposure (12, 52–55), and Abs 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 Abs that inhibit erythrocyte invasion in vitro (19, 58–64).

Dissecting the importance of specific Ags as targets of inhibitory Abs in humans is challenging because Abs to multiple Ags are coacquired through natural exposure, making it difficult to attribute inhibitory activity to specific Ags. Moreover, comparing the inhibitory activity of human Abs to isolates with different invasion phenotypes is complicated by other differences between isolates, such as polymorphisms in specific Ags that can influence the inhibitory activity of Abs (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 Abs 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 with parental parasites. Changes in the susceptibility to human inhibitory Abs 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 Abs. In addition, we evaluated the presence of Abs 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 malaria vaccine design and development.

**Materials and Methods**

**Invasion inhibition assays**

All sera were dialyzed to remove nonspecific inhibitors and run in duplicates, 1/10 dilution in two separate assays with synchronized (sorbitol used every second day for 2 wk before starting the assay) *P. falciparum* 3D7 wild-type (wt), 3D7ΔEBA175, 3D7ΔEBA140, 3D7ΔEBA181, 3D7ΔEBA175/181, 3D7ΔEBA140/175, W2meftw, and W2meftΔEBA140 parasites (67). For a subset of samples, the inhibitory effect of treated samples was confirmed by Ig purification from the same samples (67). Rabbit Abs to Msp1α were kindly provided by Brendan Crabb (The Macfarlane Burnet Institute for Medical Research and Public Health) (68). Samples from nonexposed donors were used as negative and polyclonal anti-AMA1 Abs 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% 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 nonimmune control samples in our assays, 2 SD 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 Abs against the surface of uninfected erythrocytes maintained in culture (70); very little reactivity against normal erythrocytes 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, Melbourne, Australia). All samples were obtained after written informed consent.

**Abo 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 (aa 746–1045) (19), EBA175 RIII-V W2meft and 3D7 alleles (aa 761–1271) (71), EBA181 RIII-V 3D7 allele (aa 755–1339) (18), PfRh4 3D7 allele (aa 1160–1370) (25), and PfRh2 3D7 allele (aa 2027–2533) (17), as well as schizont extract (3D7 allele) (12).

**Study population and sera samples**

Sera samples from 18 adults [median age, 47 years (range: 18–81); 22.2% male; 16.7% *P. falciparum* positive] and 53 children [median age, 8 years (range: 2–12); 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 years (range: 0–8); 41.9% male; 12.9% *P. falciparum* positive] collected in May 2003, at the start of the rainy season, which is associated with higher malaria transmission. These samples were selected from a larger cohort based on 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, nonexposed blood donors collected in May 2004 in Kilifi, and, as controls, nonexposed adult residents in Australia/U.K. (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, Melbourne, Australia). All samples were obtained after written informed consent.

**Statistical analysis**

Differences in invasion between knockout and 3D7wt were assessed using paired t tests separately for each population sample set. The association of invasion with age and parasitemic status was assessed using t tests or Mann–Whitney U test, when appropriate. Differences in the frequency of high and low invasion inhibitors with the sample set were assessed using χ² or Fisher’s exact tests, when 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 Release 16.0, 2007; SPSS, Chicago, IL) was used for statistical analysis.

**Results**

**Phenotypic variation and evasion of inhibitory Abs**

To study the function of the EBA proteins in phenotypic variation for immune escape and as targets of human inhibitory Abs, 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 and EBA175/EBA181 (61). Previously, transcriptional analysis of these lines revealed significant upregulation in PfRh4 gene expression in 3D7ΔEBA175, 3D7ΔEBA140/175, and 3D7ΔEBA175/181, compared with the 3D7 parental line (61). Otherwise, no significant changes in merozoite gene expression were noted. In 3D7ΔEBA140, there were no changes in transcriptional levels of these genes compared with those in 3D7 parental parasites.

Human serum Abs were tested for their ability to inhibit invasion of parasites with targeted disruption of EBA protein expression versus parental parasites. Differential inhibition by acquired Abs 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 Abs from individuals residing in a malaria-endemic region of Kenya were tested 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 ≥ 25% in the extent of inhibition between the comparison lines (12).

Testing human Abs in invasion inhibition assays, we found that the disruption of EBAs had a major effect on the susceptibility of parasites to inhibitory Abs, when comparing inhibition of the EBA knockout lines versus the parental parasites. This effect was seen among all sample sets examined (Figs. 1, 2, Table I). The effect was greatest with 3D7ΔEBA140 and 3D7ΔEBA175, for which 84% of samples (for both lines) gave differential inhibition of knockout versus parental parasites. Of interest, for 3D7ΔEBA140 most of the samples with differential inhibition showed greater inhibition of parental parasites than of knockout parasites in all sample sets, suggesting that Abs were directed against EBA140 and that absence of EBA140 function led to escape from inhibitory Abs for most samples. Reflecting this, the mean inhibition of 3D7ΔEBA140 by all samples was significantly lower (p < 0.05) than inhibition of 3D7 (Fig. 2). In contrast, 3D7ΔEBA175 showed a very different phenotype, with significantly greater (p < 0.05) mean inhibition of knockout compared with parental parasites by all study samples, and a substantial proportion of samples inhibited 3D7ΔEBA175 to a greater degree than 3D7 parental parasites. This finding suggests that the phenotypic change induced by loss of EBA175 function led to greater Ab inhibitory activity against the knockout parasite, possibly owing to Abs 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 Abs, with 40% of samples showing differential inhibition of 3D7ΔEBA181 compared with 3D7wt. Most samples showed greater inhibition of the knockout than of 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 inhibition of 3D7wt. Comparing the pattern of inhibitory activity against the different lines by individual samples suggests substantial diversity also exists 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; Supplemental Fig. 1).

Prior studies have shown that the function and binding affinity of EBA140 can vary substantially between different isolates and is associated with polymorphisms in EBA140 (19). Therefore, we investigated whether the genetic background of the parasite influenced the activity of human inhibitory Abs to EBA140 by testing human Abs 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 with 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 Ab activity than that 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 Abs was very similar for the two isolates (77% ± 18% in W2mefΔEBA140 and 73% ± 16% for W2mefwt). This observation 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 both of EBA140 as an Ab target and of phenotypic variation in mediating immune evasion.

These findings demonstrate that changes in the expression of EBA175, EBA140, or EBA181 substantially have an impact on the antigenic properties of merozoite invasion and the susceptibility to acquired inhibitory Abs, and that the antigenic properties of the different EBA knockout parasites exhibit differences. For controls in these studies, we tested the mutant and parental parasite lines for inhibition by Abs 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 knockout lines (Supplemental Fig. 2). Furthermore, we included serum Abs from malaria-naive donors and they gave no differential inhibition of the different parasite lines, in contrast to the findings using samples from Kenyan donors. No consistent associations between active parasitemia in donors and the level of invasion inhibition by samples were noted. However, few individuals were parasitemic at the time of sample collection, and the study was not designed to detect this association.

FIGURE 1. Differential inhibition of P. falciparum lines by serum Abs from malaria-exposed Kenyan individuals. Results show the proportion of samples (n = 130) that differentially inhibited the invasion of 3D7wt parasites compared with 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 knockout line more than the 3D7wt line is shown in green; the proportion of samples that inhibited 3D7wt more than the knockout line is shown in red.
FIGURE 2. Mean of invasion inhibition by serum Abs 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 nonexposed donors. Values represent means, and error bars show 1 SD. Differences in mean invasion of the EBA knockout lines were significant (*p < 0.05*), compared with 3D7wt parasites for all lines and sample subsets, except for 3D7ΔEBA175/140 with analysis of all samples, and Ngerenya (Ng) adults and children 1998; 3D7ΔEBA181 for analysis of Ngerenya adults and children 1998; and 3D7ΔEBA140/175 for analysis of Ngerenya adults 1998.

**Inhibition of parasites with deletion of two EBAs**

We next tested the impact of disruption of two different EBAs (double-knockout parasites) on inhibitory Abs, compared with 3D7 parental parasites (parasite lines 3D7ΔEBA140/175 and 3D7ΔEBA175/181; Figs. 1–3). Differential inhibition of the two double-knockout isolates compared with 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-knockout parasite lines, little overall difference was found in the median inhibition of the parental versus knockout parasites by all samples (Fig. 2) because similar numbers of samples gave greater or lower inhibition of the knockout parasites compared with parental. These results suggest that disruption of two EBA genes led to substantial changes in the susceptibility to inhibitory Abs; however, it did not result in a more pronounced phenotypic change compared with single EBA knockouts.

To determine whether the antigenic phenotypes of the single- and double-knockout 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 with 3D7ΔEBA140/175 double knockout, clearly indicating that the antigenic properties of these two lines and the pattern of susceptibility to inhibitory Abs 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, little differential inhibition of 3D7ΔEBA175 versus either of the double-knockout lines was noted, 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 those of the other knockout lines and from those of parental parasites.

**Relatedness between Ab 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 Abs and the different isolates. We first examined whether samples that showed less inhibition of one EBA knockout line also showed less inhibition of other EBA knockout parasites, compared with parental parasites (Table II). These analyses suggested that inhibitory Ab responses to different isolates were not strongly related. For example, of the many samples that showed less inhibition of 3D7ΔEBA140 compared with 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 with parental parasites. We next determined whether samples that showed greater inhibition of one EBA knockout line also exhibited greater inhibition of other EBA knockout parasites, compared with the parental line (Table II). Again, these inhibitory Ab 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

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

<table>
<thead>
<tr>
<th>Inhibition of 3D7wt&gt;3Δ7-KO linea</th>
<th>ΔEBA140</th>
<th>ΔEBA175</th>
<th>ΔEBA181</th>
<th>ΔEBA140/175</th>
<th>ΔEBA175/181</th>
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<tr>
<td>All Samples (n = 130)</td>
<td>107 (82.3)</td>
<td>13 (13.1)b</td>
<td>7 (5.4)</td>
<td>21 (16.2)</td>
<td>27 (20.8)</td>
</tr>
<tr>
<td>Kilifi Adults 2006 (n = 28)</td>
<td>23 (82.1)</td>
<td>N/Aa</td>
<td>4 (14.3)</td>
<td>5 (17.9)</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td>Ngerenya Adults 1998 (n = 18)</td>
<td>13 (72.2)</td>
<td>3 (16.7)b</td>
<td>0 (0)</td>
<td>3 (16.7)</td>
<td>2 (11.1)</td>
</tr>
<tr>
<td>Ngerenya Children 1998 (n = 53)</td>
<td>45 (84.9)</td>
<td>9 (17.0)b</td>
<td>2 (3.8)</td>
<td>11 (20.8)</td>
<td>21 (39.6)</td>
</tr>
<tr>
<td>Ngerenya Children 2003 (n = 31)</td>
<td>26 (83.9)</td>
<td>1 (3.2)</td>
<td>2 (6.5)</td>
<td>2 (6.5)</td>
<td>1 (3.2)</td>
</tr>
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</table>

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

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

bSample numbers were = 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.

cResults show the proportion of samples that inhibited the in vitro invasion of the 3D7 knock-out line greater than 3D7wt.
or 3D7ΔEBA175 (4.4%) compared with parental parasites; no samples showed this pattern of inhibition for both 3D7ΔEBA175 and 3D7ΔEBA140 parasites.

Overall, these analyses suggest that inhibitory Ab responses were not highly related to each other in the study samples and that each of the EBA single knockouts led to distinct antigenic or inhibitory phenotypes, as defined by the susceptibility to inhibitory Abs. However, as noted earlier, the inhibitory phenotypes and antigenic properties of the 3D7ΔEBA175/181 and 3D7ΔEBA140/175 double knockouts appeared similar to those of 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 Abs and contributes to immune escape.

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

We next aimed to understand the extent to which invasion-inhibitory Abs 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 SA-dependent (including EBA175, EBA140, and EBA181) and SA-independent (such as PfRh2 and PfRh4) pathways.

FIGURE 3. Inhibition of different *P. falciparum* lines by serum Abs from malaria-exposed Kenyan individuals. Results show the effect of serum Abs (from individuals A–H) on the invasion of the parental parasite line versus the knockout 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 nonexposed donors. All samples were tested in duplicate in two separate assays, and values represent mean ± range.

FIGURE 4. Differential invasion inhibition by human Abs of *P. falciparum* lines with single versus double gene knockouts. Results show the proportion of serum Ab samples (*n* = 130) that differentially inhibited the invasion of 3D7 parasites with a single EBA knockout, compared with 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 to a greater degree than the 3D7 double-knockout line, and in green is the proportion of samples that inhibited the invasion of 3D7 double-knockout parasite line more than the 3D7 single-knockout line. “No difference” means < 25% difference in inhibition (indicated in blue).
Reduced invasion inhibition by serum samples of the EBA knockout lines compared with parental parasites suggests the presence of Abs 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 than of the 3D7ΔEBA140 parasites, suggesting that inhibitory Abs target EBA140. The 3D7ΔEBA140 showed significantly less inhibition than did 3D7wt in all four sample sets: Kilifi adults, Ngerenya 1998 adults, Ngerenya 1998 children, and Ngerenya 2003 children (Figs. 1, 2; \( p < 0.001 \) for all differences). With other EBA knockout parasite lines, fewer samples showed this effect: 13% inhibited 3D7wt more than 3D7ΔEBA175 parasites, and only 5% inhibited 3D7wt parasites more than the 3D7ΔEBA181. This finding might suggest that EBA175 and EBA181 are less important targets of inhibitory Abs in 3D7, or that a change in invasion phenotype may occur with the loss of EBA175 or EBA181 and lead to a greater susceptibility to other inhibitory Abs that target ligands replacing the function of EBA175 or EBA181. For the double knockouts, 16% and 21% inhibited the parental more than 3D7ΔEBA140/175 and 3D7ΔEBA181/181, respectively, further supporting the conclusion that some individuals have inhibitory Abs to the EBAs, and possibly other ligands of SA-dependent invasion.

Inhibition of the EBA knockout lines more than the parental parasites suggests that inhibitory Abs may also target ligands of SA-independent invasion, such as PfRh2 and PfRh4, particularly when comparing the double knockouts with 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 with 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 than of parental parasites suggests that inhibitory Abs target these ligands, and previous studies have shown that affinity-purified human Abs 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 Abs.

**Presence of Abs to EBA and PfRh invasion ligands**

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

We also examined the relationship between Abs to EBA and PfRh ligands and differential invasion inhibition of parasites. Considering the correlation between Abs to different invasion ligands by ELISA, the ability to dissect the relationship between any Ag-specific response by ELISA and specific invasion-inhibitory activity was limited. Greater inhibition of 3D7 parental parasites than of 3D7ΔEBA140 suggested the presence of inhibitory Abs targeting EBA140. Consistent with this idea, there was a significant correlation, of moderate strength, between Abs to\n
<table>
<thead>
<tr>
<th>EBA140</th>
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<th>EBA181</th>
<th>EBA140/175</th>
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<tbody>
<tr>
<td>ΔEBA140</td>
<td>ΔEBA175</td>
<td>ΔEBA181</td>
<td>ΔEBA140/175</td>
</tr>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>11 (16.2)</td>
<td>4 (3.1)</td>
<td>2 (2.9)</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>20 (15.4)</td>
<td>2 (2.9)</td>
<td>4 (3.1)</td>
<td></td>
</tr>
<tr>
<td>25 (19.2)</td>
<td>4 (5.9)</td>
<td>3 (2.3)</td>
<td>10 (7.7)</td>
</tr>
</tbody>
</table>

Values represent number of samples and percent of total samples in brackets. \(^*\)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. \(^\dagger\)Samples that showed inhibition of the in vitro invasion of a 3D7 knock-out line that was greater than 3D7wt, for two different knock-out line comparisons.

**Table II. Relatedness between inhibitory Abs: proportion of individuals showing differential invasion inhibition of two knock-out Abs compared to 3D7wt**

![Graph showing Abs against recombinant EBA and PfRh proteins measured by ELISA in Kenyan individuals. Results show the prevalence of Abs to recombinant EBA and PfRh proteins measured by ELISA among the different sample sets included in the study. Error bars represent SE of a proportion. Ab reactivity among malaria-naïve donors was negligible.](http://www.jimmunol.org/DownloadedFrom/)
EBA140 by ELISA and the extent of differential inhibition of 3D7 parental parasites compared with 3D7ΔEBA140 (r = 0.27; p < 0.05). A similar finding was noted for EBA181, with Abs to EBA181 by ELISA being significantly correlated with the extent of differential inhibition of 3D7 parental parasites compared with 3D7ΔEBA181 (r = 0.32; p < 0.05). This observation suggests that Abs to these recombinant proteins, as measured by ELISA, are broadly reflective of functional inhibitory activity but are not strongly predictive of ligand-specific inhibitory activity of serum Abs. Few samples showed differential inhibition of 3D7 parental parasites to be greater than that of 3D7ΔEBA175 (Fig. 1); to the contrary, most samples exhibited greater inhibition of 3D7ΔEBA175 than of 3D7 parental, suggesting the presence of inhibitory Abs 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 idea, Abs to PfRh2 were significantly correlated with greater inhibition of 3D7ΔEBA175 parasites than of 3D7 parental parasites (r = 0.42; p < 0.01), especially in the Ngerenya 2003 children’s samples (r = 0.53, p < 0.01). This finding 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). A correlation also was found between Abs 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 with 3D7 parental, consistent with the greater reliance of these parasites on ligands of SA-independent invasion.

**Discussion**

Identifying major targets of protective immune responses and understanding the 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 EBA ligands are important targets of invasion-inhibitory Abs and whether variation in the expression or 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 Abs. The advantage of assays employing live parasites is that the Ags are correctly folded and presented in their native context, and Abs to all epitopes presented on the protein are measured rather than assessing Abs only to specific domains or regions, as is regularly done in ELISA assays. Our striking observations that disrupting the function of individual EBAs dramatically alters the susceptibility to human inhibitory Abs 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, when compared with 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 Abs, 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 knockout lines and the parental parasites are antigenically distinct from each other, as defined by the inhibitory profile against our panel of human Abs. This suggestion indicates that a significant level of antigenic diversity could contribute to immune evasion. Differences in the inhibitory activity of Abs 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 owing 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 Abs has not been clearly established. Studies using Abs raised in rabbits suggest that polymorphisms in the binding region of EBA175 do not have a major impact on the invasion-inhibitory activity of Abs (75, 76). However, we did demonstrate in this article that the genetic background of the parasite influences the importance of EBA140 as an Ab target and its role in phenotypic variation to mediate immune evasion. Although EBA140 appeared to be an important target of inhibitory Abs with the 3D7 isolate, it appeared much less important in the W2mef isolate. This finding 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 with 3D7 EBA140, even though the expression of protein appears similar between isolates (77). The EBA140 sequence differs in three amino acids between 3D7 and W2mef (in the F1 domain). Furthermore, Abs to EBA140 generated in rabbits gave much greater inhibition of invasion with 3D7 parasites than with W2mef parasites (19, 77), consistent with our findings in this article using human Abs, 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 Abs. A majority of samples showed reduced inhibition of 3D7ΔEBA140 compared with 3D7wt, suggesting inhibitory Abs 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, and PfRh4 (61), indicating that the differential inhibition seen is not due to that action of Abs against other EBAs or PfRh's. Fewer samples demonstrated reduced inhibition of 3D7ΔEBA175 compared with 3D7wt, reflecting the presence of inhibitory Abs to EBA175 in the population. It is possible that EBA175 is a less important target of inhibitory Abs with the 3D7 parasite line we used, compared with EBA140. Alternatively, the loss of EBA175 may lead to a wider change in the invasion phenotype and to a greater reliance on other invasion ligands, such as EBA140 or PfRh ligands that are prominent targets of inhibitory Abs. The importance of the EBAs as targets of inhibitory Abs was supported by results showing that Abs to recombinant EBAs by ELISA were highly prevalent in the population, and there were some significant correlations between Abs to recombinant EBA140 and EBA181 proteins and differential inhibition of parental versus knockout parasite lines. However, our results suggest that measuring Abs to recombinant proteins by ELISA cannot be relied upon as a proxy for functional Ab activity. Future studies with full-length or native proteins may help us understand the relationship between Ab levels and function; however, standard immunoassays cannot account for Ab affinity and epitope specificity, which are likely to be important for functional activity. This lack 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 Abs. This idea was demonstrated by greater inhibition by human Abs of 3D7ΔEBA175 (or EBA175/140 and EBA175/181
PfRh ligands, which may explain why we did not observe significant inhibition of invasion at physiologically relevant concentrations (53). Affinity-purified Abs to PfRh4 from malaria-exposed donors can and PfRh4 are associated with protective immunity, and that affinity-purified Abs to PfRh4 from malaria-exposed donors can inhibit invasion at physiologically relevant concentrations (53, 54). Many individuals may have functional Abs to both EBAs and PfRh ligands, which may explain why we did not observe significant differential inhibition of 3D7DEBA140 or 3D7DEBA140/175 compared with 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 or 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 Ags to overcome the parasite’s capacity for immune evasion, such as a combination of EBA and PfRh ligands (12). Whereas most research on the EBAs has focused on EBA175 as a potential subunit vaccine candidate, our results highlight EBA140 as an important target of inhibitory Abs, and this Ag needs to be further studied as a potential vaccine candidate. Recent reports suggest that vaccine-induced Abs raised in rabbits against PfRh5, or its binding partner, contribute to immune evasion and that the EBAs are important targets of human invasion-inhibitory Abs. The importance of the EBAs supports their development as promising vaccine candidates; however, the demonstration in this article of their limited level of polymorphism (4). Our results suggest that the EBAs contribute to immune evasion and that the EBAs are associated with protection from clinical malaria. Infect. Immun. 76: 2240–2248.

The authors have no financial conflicts of interests.

Disclosures

The authors have no financial conflicts of interests.

References


Supplementary Figure 1:

**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.

Supplementary Figure 2:

**Inhibition of different *P. falciparum* lines by anti-MSP1-19 and heparin.** 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.