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Functional Analysis Reveals Geographical Variation in Inhibitory Immune Responses Against a Polymorphic Malaria Antigen

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Background. *Plasmodium falciparum* reticulocyte-binding protein homologue 2b (PfRh2b) is an invasion ligand that is a potential blood-stage vaccine candidate antigen; however, a naturally occurring deletion within an immunogenic domain is present at high frequencies in Africa and has been associated with alternative invasion pathway usage. Standardized tools that provide antigenic specificity in in vitro assays are needed to functionally assess the neutralizing potential of humoral responses against malaria vaccine candidate antigens.

Methods. Transgenic parasite lines were generated to express the PfRh2b deletion. Total immunoglobulin G (IgG) from individuals residing in malaria-endemic regions in Tanzania, Senegal, and Mali were used in growth inhibition assays with transgenic parasite lines.

Results. While the PfRh2b deletion transgenic line showed no change in invasion pathway utilization compared to the wild-type in the absence of specific antibodies, it outgrew wild-type controls in competitive growth experiments. Inhibition differences with total IgG were observed in the different endemic sites, ranging from allele-specific inhibition to allele-independent inhibitory immune responses.

Conclusions. The PfRh2b deletion may allow the parasite to escape neutralizing antibody responses in some regions. This difference in geographical inhibition was revealed using transgenic methodologies, which provide valuable tools for functionally assessing neutralizing antibodies against vaccine-candidate antigens in regions with varying malaria endemicity.

Keywords. growth inhibition; invasion; PfRh2b; transgenic; vaccine.

Malaria caused by *Plasmodium falciparum* represents a major public health challenge with at least 400 000 deaths annually, occurring primarily in young children and pregnant women [1]. The process of merozoite invasion is a critical step in the *Plasmodium* life cycle, and the multiple ligand–receptor interactions involved in invasion influence parasite virulence and disease severity [2]. To establish infection within the human host, the merozoite faces 2 selective pressures: erythrocyte receptor polymorphism and the humoral immune response. Such selective pressures are manifested both in variant expression of parasite ligands and in sequence polymorphisms in the genome.

With the rise in antimalarial drug resistance, an effective vaccine is ever more critical. While most vaccines currently under development target the sporozoite stage of the life cycle, there has been a recent resurgence in interest in blood-stage vaccines targeting merozoite invasion ligands [3–6]. In assessing vaccine candidates, it is important to identify polymorphisms under selection and determine whether such polymorphisms contribute to altered receptor binding (eg, erythrocyte-binding antigen [EBA]–181) [7] or immune evasion (eg, apical membrane antigen 1 [AMA-1]) [8].

The *P. falciparum* reticulocyte-binding protein homologue 2b (PfRh2b) is an important sialic acid–independent invasion ligand [9] and is a member of a multigene family of reticulocyte binding–like (RBL) proteins involved in the commitment step of invasion. PfRh2a and PfRh2b are identical in sequence for their first N-terminal 2700 amino acids, yet diverge in their c-terminal 400–500 amino acids—a region that includes variable heptad repeats, a unique ectodomain that differs between PfRh2a and PfRh2b, a transmembrane domain, and a cytoplasmic

tail [10, 11]. The PfRh2a/2b erythrocyte-binding domain has been mapped to the N-terminus of the protein; however the c-terminal unique region of PfRh2a has been shown to have some erythrocyte-binding activity as well [12]. Variation in PfRh2b expression is associated with alternative invasion pathway use in both laboratory and field isolates [13, 14]. A large polymorphism in the c-terminal unique ectodomain region of PfRh2b was described in the *P. falciparum* line T996 [15] and was identified in a large percentage of field isolates in Senegal [16]. In 2 studies in Senegal, the PfRh2b deletion was associated with invasion pathway utilization [16, 17]. The deletion allele is highly prevalent within Africa and is proposed to be under strong immune selection [18].

Population genetic analyses can infer the type of selection acting at these loci [19, 20], but genetic methodologies can be employed to precisely determine the role of a given polymorphism. By using transgenic methodologies, a specific polymorphism can be studied in an isogenic background, controlling for unrelated polymorphisms in many antigens. In this study, we use genetic replacement to test the role of the PfRh2b deletion polymorphism in both invasion pathway utilization (receptor binding) and immune evasion in 3 regions with dramatically different levels of malaria endemicity.

METHODS

Study Subjects and Samples
Patients were recruited in Thiès, a region in Senegal with low *P. falciparum* endemicity and seasonal malaria transmission (entomological inoculation rate [EIR] = 1–5 infectious bites/person/year) [21] in 2008–2009, when parasite positivity rate in children between 2–10 years of age ($PfPr_{2-10}$) has been modeled to be 6% [22]; and in Mlandizi, a region of Tanzania of high malaria endemicity and perennial transmission (EIR = 200) [23] in 2003–2004, when $PfPr_{2-10}$ has been modeled to be 23%–26% [22]. In both Thiès and Mlandizi, patients diagnosed with uncomplicated malaria were enrolled in the study after informed consent was obtained. In Kenieroba, Mali, an area of high malaria endemicity and perennial transmission (EIR > 100) (personal communication, Adama Dao and Nafomon Sogoba, International Center for Excellence in Research (ICER)/FMOS/USTTB, Bamako, Mali), adults known to be highly malaria-exposed were recruited from May 2013 to May 2014, when $PfPr_{2-10}$ has been modeled to be 42%–43% [22], and donated blood for plasma collection after informed consent was obtained. For the purposes of population transmission comparisons, here we consider low transmission to be EIR < 10; medium transmission, EIR = 10–100; and high transmission, EIR > 100 [24].

Transgenic Parasite Lines
The PfRh2b deletion single crossover plasmid was generated by amplifying the c-terminal 1.3 kilo base pairs from a sequenced Senegalese field strain genomic DNA containing

the PfRh2b deletion (and all other sequence identical to 3D7). The PfRh2b wild-type replacement parasite line (1A) was generated and characterized previously [25]. PfRh2b knockout parasites (3D7Δ2b, C2; W2MefΔ2b, C23) were also described previously [9, 25]. Details of transgenic parasite line characterization (Western blots, immunofluorescence microscopy, and quantitative polymerase chain reaction [qPCR]) are included in Supplementary Methods. Here, we refer to PfRh2b wild-type replacement parasite line (1A) as "WT," the PfRh2b deletion single crossover parasites as "DEL," and 3D7Δ2b, C2 and W2MefΔ2b, C23 parasites as "KO." Two independent transgenic deletion clones were characterized in each genetic background: in 3D7, DEL A (clone A12), and DEL B (clone H11); and in W2Mef, DEL C (clone A10) and DEL D (clone C10).

Invasion Assay
Invasion assays were performed as previously described [26, 27]. Additional details can be found in Supplementary Methods.

Invasion Phenotype Measurements
Synchronized ring-stage parasites were plated at 1% parasitemia and monitored by microscopy at the ring (T = 0 hours), trophozoite/early schizont (T = 40 hours), late schizont/reinvaded ring (T = 48 hours), and ring/trophozoite (T = 56 hours) stages. Parasite multiplication rate was calculated as follows: (final parasitemia)/(initial parasitemia). The selectivity index was counted as previously described [28, 29]. Additional details can be found in Supplementary Methods. Average merozoite numbers (Av Mero) were counted for late-stage segmented schizonts only (T = 48), and 100 schizonts were counted per experiment to determine the average merozoite number. Invasion efficiency was calculated as described [30]: Invasion efficiency = (final parasitemia)/[(average merozoite number) × (initial parasitemia)].

Invasion Inhibition Assays
Invasion inhibition assays were performed similarly to invasion assays with minor modifications. Wild-type 3D7 parasites and DEL A (3D7-PfRh2b deletion) were used and invasion inhibition was measured into untreated erythrocytes (Roswell Park Memorial Institute medium) as well as neuraminidase-treated erythrocytes (Nm). Parasites were plated at 0.25% parasitemia in 2% hematocrit in 25 μL final volume. Protein A–purified antibodies from rabbits immunized with the unique domain of PfRh2b [31, 32] were added to synchronized ring-stage parasites at a final concentration of 250 μg/mL and incubated for 1 cycle of reinvasion.

Growth Inhibition Assays
Growth inhibition assays were performed as previously described [33], using protein G-purified total immunoglobulin G (IgG) from Thiès, Senegal (n = 39), Mlandizi, Tanzania (n = 33), and Kenieroba, Mali (n = 27), at a final concentration of 10 mg/mL and transgenic parasites: WT, DEL A, and

KO. Transgenic parasites were continuously grown under WR-99210 pressure.

RESULTS

Generation of Transgenic Parasites to Test the Role of the PfRh2b Deletion Allele

To determine the specific role of the PfRh2b deletion polymorphism on invasion pathway, efficiency, and immune evasion, we generated transgenic parasite lines that harbored the PfRh2b deletion (DEL$_{3D7}$), or the PfRh2b full-length allele (WT) [25], in a genetically identical 3D7 parental background (Supplementary Figure 1). We introduced the PfRh2b deletion into the PfRh2b locus by homologous recombination and single crossover (Supplementary Figure 1A). We confirmed disruption of the endogenous locus by Southern blot (Supplementary Figure 1B) and sequencing (Supplementary Figure 1D). We also created transgenic PfRh2b deletion (DEL$_{W2Mef}$) parasites in the W2Mef background (Supplementary Figure 1C).

The PfRh2b Deletion Does Not Alter Gene Expression, Protein Expression, Proteolytic Cleavage, or Localization of Other Invasion Ligands

To determine whether the deletion influences gene expression, we performed qPCR on complementary DNA prepared from schizont-stage ribonucleic acid for 3D7, WT, DEL A, and DEL B. The ΔΔCT values ranged from −0.72 to 1.98, and revealed no significant fold change for all transgenic lines relative to 3D7 (Figure 1A). To determine whether the deletion might affect protein expression and cleavage of other important invasion ligands, we performed Western blots of both schizont and invasion supernatant extracts (Figure 1B) for 3D7, WT, and DEL A and DEL B clones, and probed with anti-PfRh2a and anti-PfRh2b antibodies. We found no difference in expression levels of PfRh2a and PfRh2b (other than the size difference in the PfRh2b protein due to the deletion). Additionally, we found no change in apical merozoite localization of PfRh2b, adjacent to the rhoptry bulb, as indicated by partial colocalization with RhopH3 (Supplementary Figure 1D).

The PfRh2b Deletion Does Not Alter Invasion Pathway or Measurements of Invasion Efficiency

While past reports from Senegal have shown that the PfRh2b deletion was mildly associated with sialic acid–dependent invasion [16], high trypsin-resistant invasion, and marginally, low trypsin/chymotrypsin-resistant invasion [17], we observed no difference in invasion pathway utilization between the 3D7 and DEL parasite lines for all enzyme treatments tested, in contrast to a striking difference in invasion pathway compared to the PfRh2b knockout (KO) (Figure 1C). The most significant differences were observed into chymotrypsin treated (3D7, $P = .0001$; DEL A, $P = .0001$; DEL B, $P = .0001$; relative to KO) and low trypsin/chymotrypsin-treated cells (3D7, $P = .0028$; DEL A, $P = .0058$; DEL B, $P = .0346$; relative to KO), the characteristic

enzyme treatments defining the PfRh2b receptor. Thus, the deletion does not mimic a PfRh2b "loss of function" phenotype, as is observed with KO compared to 3D7. The same result was found in the W2Mef genetic background (Supplementary Figure 2).

To address whether the PfRh2b deletion may confer a fitness advantage in the absence of in vivo selective pressures, we compared multiple measures of invasion efficiency for the 3D7, WT, and DEL transgenic lines. In 3 independent experiments, erythrocytic cycle length (Figure 1D), parasite multiplication rate, selectivity index, and invasion efficiency of the deletion strains were not found to differ significantly from 3D7 and WT (Table 1). There was a small difference in the average merozoite number per schizont (Av Mero) for the 2 deletion clones (DEL A $P = .01$ and DEL B $P = .02$ relative to 3D7; DEL A $P = .005$ and DEL B $P = .008$ relative to WT) (Figure 1E); however, this difference was not manifested in a difference in invasion efficiency, which takes merozoite number into account (Table 1).

The PfRh2b Deletion Influences Parasite Growth Rate Over Time

While subtle differences in invasion efficiency may not be significant in a single round of parasite replication, they can amplify over multiple rounds of growth. We performed in vitro competition experiments, similar to those described by others [34], between the WT and DEL clones (DEL A or DEL B) over 4 weeks, corresponding to 14 cycles of parasite replication. We observed that the DEL A and DEL B clones outgrew the WT controls at a rate of 8.4% per cycle for DEL A and 18.9% per cycle for DEL B, with a combined 13% per cycle (Figure 1E), implying that the deletion influences invasion over time.

The PfRh2b Deletion Allows Parasites to Escape the Effect of PfRh2b-Specific Inhibitory Antibodies

The 2 main selective pressures driving polymorphism in vivo are erythrocyte-receptor polymorphism and humoral immunity. We tested the hypothesis that parasites with the deletion can escape inhibitory antibodies by performing antibody inhibition assays in the presence of antibodies against the PfRh2b unique region, which were previously shown to inhibit invasion of parasite isolates invading by a trypsin-resistant pathway [32]. IgG from rabbits immunized with the PfRh2b unique domain did inhibit wild-type (3D7) parasites modestly, yet significantly more than DEL parasites ($P = .0419$), but only when the parasites were limited to a sialic acid–independent pathway through treatment with neuraminidase (Supplementary Figure 3).

Population-Specific Development of PfRh2b Allele-Transcendent Immune Responses

To determine the inhibitory potential of naturally acquired IgG from malaria-endemic populations, we employed the highly standardized growth inhibition assay [33] using IgG from children in a highly endemic region of Tanzania, from adolescents and adults in a low-endemicity region of Senegal, and adults in
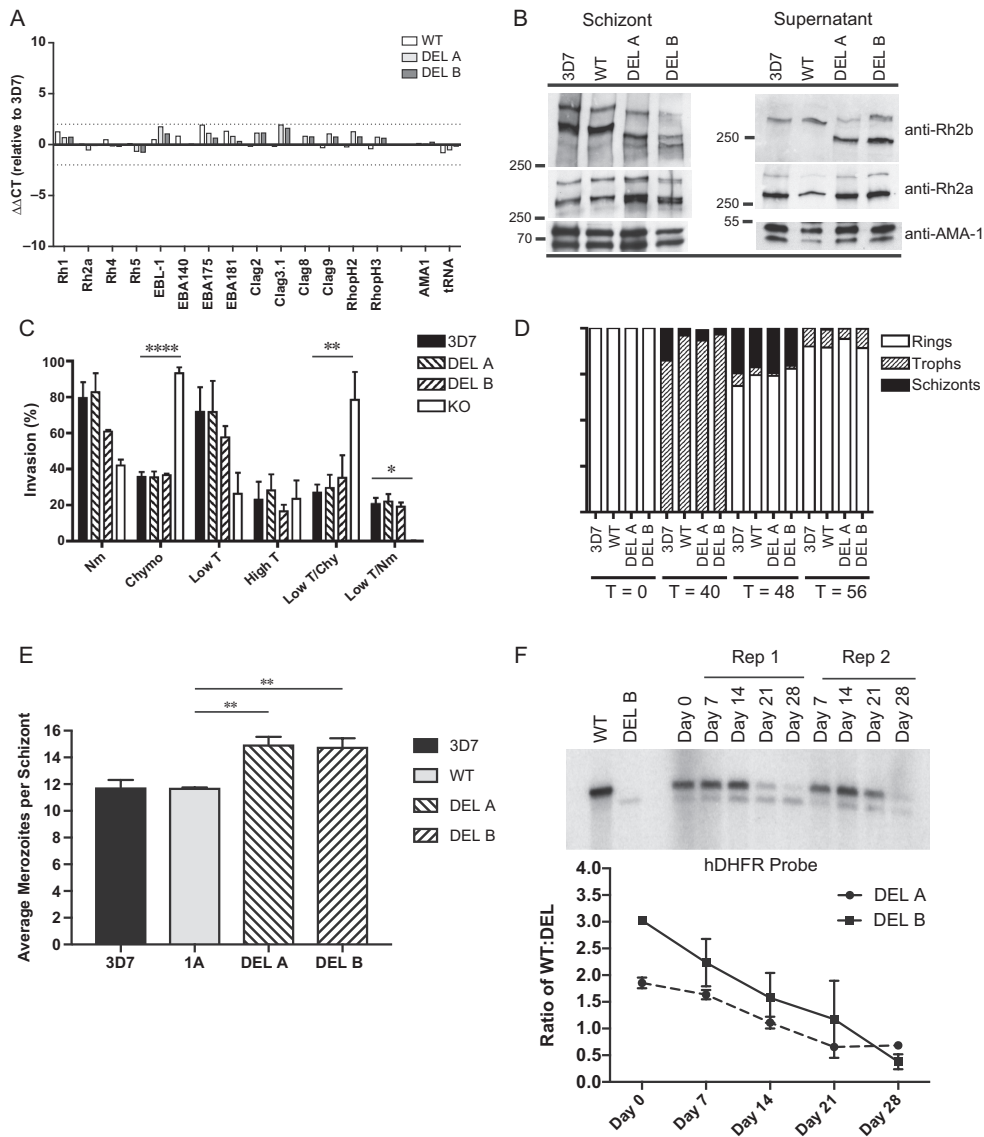
Figure 1. The PfRh2b deletion does not affect alternative invasion pathway utilization but shows a competitive growth advantage over time. *A,* Quantitative PCR was employed to determine differences in invasion gene expression between DEL clones and WT control. CT values were compared relative to AMA-1 (ΔCT). Data are expressed as fold change (ΔΔCT) values relative to 3D7. Clag 3.2 is not shown as it is not expressed in the 3D7 clone used. *B,* Western blots of synchronous schizont material and invasion supernatants were performed. Samples were adjusted relative to the schizont stage–specific proteins PfAMA-1 for both schizont blots and supernatant blots. In schizonts, expected size of PfRh2b full-length protein (3D7, WT) is 382 kDa, which after proteolytic processing results in a 297 kDa cleavage product [45]; whereas the Deletion (DEL) results in a shorter protein which runs approximately 360 kDa with the cleavage product at 275 kDa. For PfRh2a, the unprocessed form runs at 370 kDa with a 285-kDa processed form. In supernatants, further processing results in an additional processed form of 290 kDa for PfRh2b (3D7, WT), 268 kDa (DEL), and 279 kDa for PfRh2a [45]. *C,* Invasion assays were performed to determine the erythrocyte receptor utilization for DEL clones compared to wild-type 3D7 and KO (3D7ΔPfRh2b). Percent invasion into enzyme-treated erythrocytes was calculated relative to invasion of the same parasite line into RPMI-treated erythrocytes. Assays were repeated 5 times, in triplicate, except for KO and DEL B (which were repeated twice, in triplicate). Values shown are the mean of all experiments; error bars represent SD. Overall significance is assessed by 1-way ANOVA; differences between columns are assessed with Dunnett test for multiple comparisons. Asterisks indicate significant differences (*$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$). KO invasion into lowT/Nm was tested, but is undetectable. *D,* Detailed measurements of invasion and growth were performed for PfRh2b deletion and control strains for 3 independent experiments, plated in duplicate. Erythrocytic cycle duration was measured for the 2 PfRh2b deletion (DEL A and DEL B) and control strains (3D7, WT) over 1 cycle. Stages were quantified morphologically at T = 0, T = 40, T = 48, and T = 56 hours postinvasion. A total of 300 parasitized cells were counted per time point; the average of the 3 experiments, plated in duplicate, is shown. *E,* Average merozoite number per schizont was calculated for transgenic parasites and controls. Data shown are from 3 independent experiments. Means and 95% CIs are shown. Overall significance is assessed by 1-way ANOVA; differences between columns are assessed with Dunnett test for multiple comparisons. Asterisks indicate significant differences (*$P < .05$; **$P < .01$; ***$P < .001$). *F,* Competitive growth assays were conducted in which DEL and WT were mixed in equal proportions and kept in in vitro culture for 1 month, with weekly gDNA harvests. Relative proportions of DEL and WT were assessed by Southern blot hybridization using a probe to human DHFR. A representative experiment is shown with DEL B and WT. Relative ratios of WT compared to DEL over time were calculated for both DEL A and DEL B clones, with 2 independent experiments per clone; error bars represent the range of the 2 experiments. Abbreviations: ANOVA, analysis of variance; CIs, confidence intervals; CT, cycle threshold; DEL, PfRh2b deletion single crossover parasites; DHFR, dihydrofolate reductase; gDNA, genomic DNA; kDa, kilodalton; KO, 3D7Δ2b, C2 parasites; lowT, low trypsin-treated erythrocytes; Nm, neuraminidase-treated erythrocytes; PCR, polymerase chain reaction; PfRh2b, *Plasmodium falciparum* reticulocyte-binding protein homologue 2b; RPMI, Roswell Park Memorial Institute; T, trypsin-treated erythrocytes; WT, PfRh2b wild-type replacement parasite line (1A).

Table 1. Measures of Invasion Efficiency for PfRh2b Allele Transgenic Strains

	PMR	SI	IE
Strain (mean, CI)			
3D7	5.72 (4.25, 7.17)	8.93 (4.48, 13.39)	49.17 (26.25, 72.08)
WT	4.37 (3.02, 5.72)	12.70 (7.20, 18.20)	37.30 (25.50, 49.10)
DEL A	7.21 (3.02, 11.40)	9.10 (6.32, 11.88)	48.32 (19.95, 76.69)
DEL B	5.80 (2.45, 9.16)	12.24 (5.52, 18.97)	39.69 (12.40, 66.98)
1-way ANOVA	$P = .0927$	$P = .1023$	$P = .3679$

PMR, SI, and IE are calculated for transgenic parasites and controls. Data shown is from 3 independent experiments. Means and 95% CIs are shown. Overall significance is assessed by 1-way ANOVA.

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; DEL, PfRh2b deletion single crossover parasites; IE, invasion efficiency; PfRh2b, *Plasmodium falciparum* reticulocyte-binding protein homologue 2b; PMR, parasite multiplication rate; SI, selectivity index; WT, PfRh2b wild-type replacement parasite line (1A).

a highly endemic region in Mali. We observed strain-specific differences in growth inhibition for WT, DEL A, and KO strains (Figure 2A). When stratified by endemic region, in Tanzania, inhibition was significantly different for all strains, with WT inhibited less than DEL A, and DEL A inhibited less than KO (WT < DEL < KO) (Figure 2B). In contrast, in Senegal, the KO is inhibited less than WT and DEL A (WT = DEL > KO) (Figure 2C). In Mali, for adults with significant malaria exposure, while overall inhibition was high, there were no differences between alleles (WT = DEL = KO), suggesting that the PfRh2b allelic differences do not account for overall differences in inhibition, and a more strain-independent immunity has been achieved (Figure 2D). Because age distributions of the 3 geographic populations were not similar, we conducted a multivariate analysis with both age and site as covariates. For inhibition
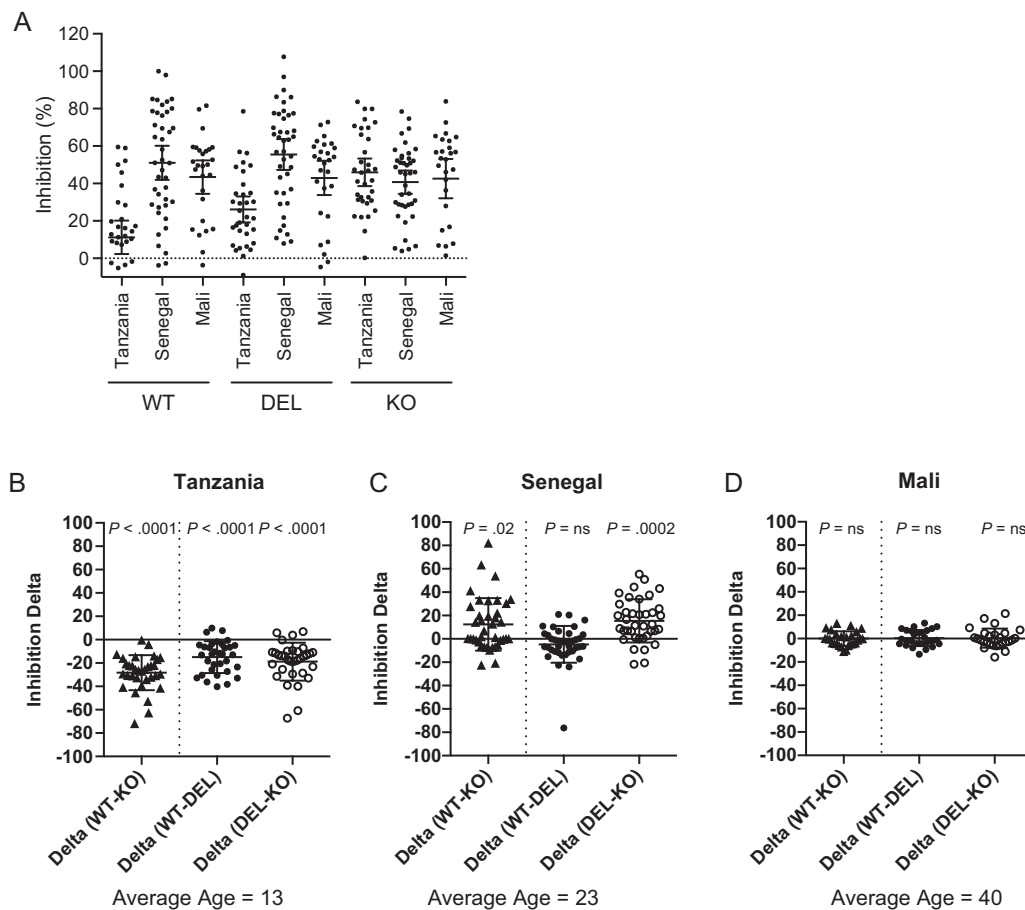

Figure 2. PfRh2b deletion reveals region-specific differences in immune-mediated inhibition of invasion. *A*, Purified IgG from Tanzania, Senegal, and Mali were used in highly standardized GIAs with transgenic parasite lines (WT, DEL A, and KO). Inhibition means and 95% CIs are shown. *B–D*, Inhibitory profiles in each endemic region show differences by strain. Inhibition is displayed as "Inhibition Δ" showing the difference between strains. On the left of each graph is the Inhibition Δ for WT-KO, with any differences representing differences in inhibition of the PfRh2b (WT) and non-PfRh2b (KO) invasion pathways; in the middle, the Inhibition Δ are shown for specific allelic inhibition of either PfRh2b full-length (WT) compared to PfRh2b deletion (DEL); and on the right, Inhibition Δ are shown for specific allelic inhibition of either PfRh2b deletion (DEL) compared to KO. Significant *P* values from comparing inhibition of WT to DEL A, and DEL A to KO, using nonparametric-paired *t* tests (Wilcoxon matched pairs) are indicated. Abbreviations: CIs, confidence intervals; DEL, PfRh2b deletion single crossover parasites; GIAs, growth inhibition assays; IgG, immunoglobulin G; KO, 3D7Δ2b, C2 parasites; ns, not significant; PfRh2b, *Plasmodium falciparum* reticulocyte-binding protein homologue 2b; WT, PfRh2b wild-type replacement parasite line (1A).

of WT parasites, the age effect was significant at $P = .0083$; the site effect was significant at $P < .0001$ (Senegal > Mali, $P = .0432$; Senegal > Tanzania, $P < .0001$). For inhibition of DEL parasites, the age effect was marginally significant at $P = .0478$; the site effect was significant at $P < .0001$ (Senegal > Mali, $P = .0102$; Senegal > Tanzania, $P = .0002$). For inhibition of KO parasites, inhibition was high across all ages and sites, and no significant age effect ($P = .0983$) or site effect ($P = .3728$) was observed.

A Model of PfRh2b-Dependent and -Independent Inhibitory Immune Responses

Transgenic parasites expressing allelic variants of a key invasion ligand in an isogenic background allow for (1) a specific determination of the role of a given allele on the increase or decrease in immune neutralization, and (2) the comparison of the role of a given allele on the overall level of inhibitory immunity. These 2 situations are observed using selected individual samples (Figure 3A) and total samples from each African population (Figure 3B). Both allele-specific and allele-independent inhibitory patterns are observed, at different frequencies in each population. In situation A, there is no significant difference in immune inhibition between the 3 transgenic strains, implying that all alleles of PfRh2b (WT, DEL A, and KO) are recognized equivalently. This can be seen in sample 61 where there is very little inhibition, as well as in sample 31 where inhibition is high but is not due to PfRh2b (Figure 3A, situation A). This inhibitory situation is rarely observed in Tanzania, found more frequently in Senegal, and represents the majority situation in Malian samples (Figure 3B). In situations B, C, D, E, and F, different kinds of allele-specific inhibitory profiles are revealed (Figure 3A, situations B–F). Situation C, which is only observed in Senegal, represents high inhibition of PfRh2b WT, partial escape from inhibition with the deletion, and further escape when PfRh2b is absent. Situations E and F represent significantly greater inhibition of the KO than the WT and DEL, implying inhibition of a non-PfRh2b pathway. By assessing the invasion inhibition
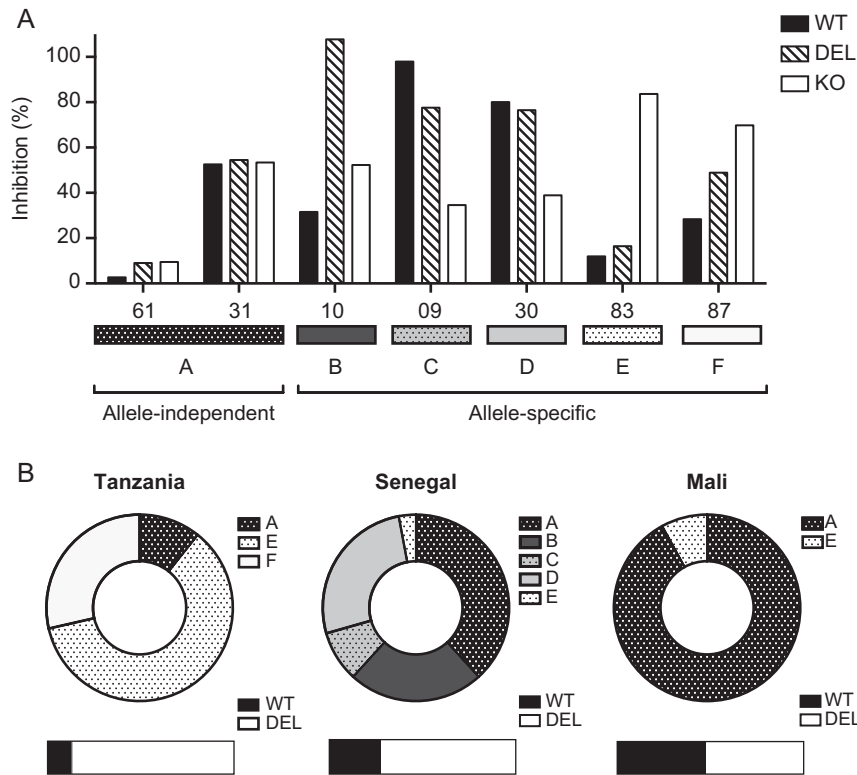

Figure 3. Model of PfRh2b-dependent and -independent inhibitory immune responses. Examples of immune neutralization that is either PfRh2b allele-specific or allele transcendent is illustrated using individual samples from Senegal (*A*) and all samples from Tanzania, Senegal, and Mali (*B*). A significant difference is considered greater than 15% difference in inhibition, the degree of difference tolerated in replicates of the same strain in the GIA. *A*, Situation A shows no significant difference in immune inhibition between the 3 transgenic strains, whether low inhibition or high inhibition, termed allele-independent immunity. Situations B–F demonstrate differences in inhibition specifically due to the allelic form of PfRh2b (allele-specific immunity). Situation B shows increased inhibition of DEL relative to the WT and KO. Such a result could indicate that the deletion in the protein is revealing an alternative epitope of PfRh2b. In situation C, there is an "immune escape" phenotype observed for both the PfRh2b deletion as well as the KO, implying that the differences in inhibition observed are specifically due to the allele of PfRh2b present. Situation D shows "immune escape" for the PfRh2b KO only. Situation E shows enhanced inhibition of both the WT and the deletion allele, and situation F shows incremental increases in inhibition from WT, to PfRh2b deletion, to PfRh2b KO. *B*, The fraction of the total for each type of inhibitory situation is shown for each population. The population genetic prevalence of the PfRh2b full-length and PfRh2b deletion alleles are shown below as bars. Abbreviations: DEL, PfRh2b deletion single crossover parasites; GIA, growth inhibition assay; KO, 3D7Δ2b, C2 parasites; PfRh2b, *Plasmodium falciparum* reticulocyte-binding protein homologue 2b; WT, PfRh2b wild-type replacement parasite line (1A).

using allele-specific tools, the inhibitory potential of naturally acquired IgG can be classified as allele-specific or allele-independent, having important implications for vaccine design and candidate prioritization in diverse geographical regions with varying malaria endemicity.

DISCUSSION

In the evaluation of novel blood-stage vaccine-candidate antigens, it is critical to consider not only the impact of variant expression of invasion ligands but also the degree to which genetic diversity in amino acid sequence and structure influences immune inhibition. Associative studies implicated the PfRh2b deletion in alternative invasion pathway utilization [16, 17], and population genetic analyses suggest that it may be selected for by strong directional immune pressure [18]. Further, the PfRh2b locus has been implicated in continental-level natural selection, with the coding region deletion present in Africa and a larger 5.9 kilo base pair deletion (spanning the c-terminus and into the neighboring PfRh6 pseudogene) identified in South East Asia [35]. Generating the PfRh2b deletion in an isogenic parasite background made it possible to determine the role of this polymorphism in either invasion pathway utilization or immune evasion, independently from other polymorphisms. Using these transgenic parasites, we found that the deletion does not influence invasion pathway as previously proposed. It is possible that this result is due to the deletion being linked with other polymorphisms in field-isolated parasites that influence the invasion pathway. Alternatively, it is possible that the deletion does not alter the invasion pathway in the 3D7 genetic background used in generating the transgenic lines. However, we tested PfRh2b deletion transgenic clones generated in the W2Mef genetic background and similarly found no influence of the deletion on invasion pathway (Supplementary Figure 2). Short-term measures of cycle length and invasion efficiency were not significantly different for the deletion parasites compared to controls; however, an increased growth rate over time was observed for deletion lines. This enhanced growth rate over multiple cycles of invasion could provide the parasites with a selective advantage in vivo in either the presence or absence of neutralizing antibodies.

We observed that the PfRh2b deletion allows parasites to escape the inhibitory effect of PfRh2b c-terminal unique domain-specific antibodies. When total IgG from malaria-endemic regions is tested, various inhibitory scenarios are observed, including PfRh2b-independent inhibition (situation A), enhanced inhibition of the PfRh2b deletion (situation B), immune escape for parasites with the PfRh2b deletion (situation C), immune escape of the KO (situation D), and enhanced inhibition of the KO, implying inhibition of a non-PfRh2b pathway (situations E and F) (Figure 3). The overall pattern of allele-specific versus allele-independent inhibitory immune responses varies by population (Figure 3B), likely due to differences in invasion pathways used in each population [13, 16,

17], differences in exposure to particular allelic variants, as well as the cumulative frequency of malaria exposure in the individual. Having the ability to distinguish these different inhibitory situations sheds light on the mechanisms of anti-PfRh2b immunity generated in natural populations. While both site- and age-specific differences were observed for WT and DEL parasites, KO parasites showed consistently high levels of inhibition in all sites. The PfRh2b knockout (KO) parasite is more dependent on a neuraminidase-sensitive, chymotrypsin-resistant invasion pathway. Equivalent inhibition of KO across age groups and sites, and with high inhibition (even in younger children), might imply that antibodies against sialic acid–dependent, chymotrypsin-independent pathways (such as those used by EBA-175) might be generated first, as has been observed using serum samples from Kenya [36], and has been suggested by others who found an inverse correlation between EBA-175 expression and age in The Gambia, presumably due to immune selection [37]. An alternative explanation is that a KO of any invasion ligand results in a decrease in the overall repertoire of inhibitable ligands—making the strain more susceptible to inhibition in general [36, 38]. This could be possible in the case of the PfRh2b KO, as there is no upregulation of alternative ligands, just a revealing of alternative ligands without a compensatory change [39].

Besides invasion pathway, another factor influencing geographic differences in PfRh2b allele-specific immune responses is the population prevalence of the wild-type or deletion alleles. While the population prevalence of the deletion is relatively high within African countries, it varies dramatically in Latin America and Southeast Asia [18]. It is possible that in regions where more inhibitory responses exist against PfRh2b, the deletion allele may be selected to higher frequencies. Studies in Papua New Guinea have implicated antibodies against PfRh2b in protection from malaria [40] and, intriguingly, there is a high frequency of the deletion in not-too-distant Malaysia (Sabah). Alternatively, inhibitory antibodies against the deletion could simply reflect greater prevalence, thus greater immune exposure, to the deletion allele. In the populations studied here, while Tanzania and Senegal both have high prevalence of the deletion (87% and 72%, respectively; Figure 3B [18],), Mali has a deletion prevalence of 52.5% (Figure 3B), meaning infected individuals in Mali are equally likely to be infected with the full length compared to the deletion. Interestingly, Mali shows few allele-specific differences in inhibition, whereas dramatic differences are observed in both Tanzania and Senegal, regions with more variation in allelic frequency; and in the case of Tanzania, high variant expression and alternative invasion pathway usage [13]. While the data here might tend to favor the argument that dramatic differences in the prevalence of the allele in the population will result in allele-specific inhibitory antibodies directed against that allele, further studies from diverse geographic locations with varying endemicities are needed to determine the

association between PfRh2b deletion frequency and immune pressure at this locus.

Interest in pursuing blood-stage vaccines for *P. falciparum* is growing, and the PfRh and PfEBA families of invasion ligands are potential candidates, whether singly or in combination [38, 41]. However, dynamics such as variant expression and polymorphism will be very important to consider before moving forward with such vaccines. Interestingly, the malaria field has not rapidly adapted transgenic approaches to assess immune inhibition and invasion [8, 36, 42, 43]. To date, only 1 study has used allelic replacement to study the impact of naturally acquired polymorphisms in the context of immune inhibition [8]. While some have used chimeric parasites or knockouts to assess immune inhibition from naturally acquired antibodies from a single-endemic country [42, 44], none have done so comparatively in different malaria-endemic countries. Our study combines transgenic parasites expressing naturally occurring PfRh2b alleles with naturally acquired IgG to assess the PfRh2b allelic contribution to invasion inhibition comparatively from 3 malaria-endemic countries. From this data, we can conclude that rather than having a role in alternative invasion pathway utilization, the deletion could have been selected through population-specific immune-mediated selection. The genetic methodologies described here provide a method for assessing inhibitory immune responses to allelic variants in vitro and facilitate comparison of the specific contribution of allele-specific antibodies to the overall inhibitory effect—a critical aspect of prioritizing vaccine candidates in the face of genetic diversity.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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